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IMMUNOPEROXIDASE LOCALIZATION OF *UREAPLASMA UREALYTICUM* ANTIGEN IN CELL CULTURE AND IN TISSUES OF PREGNANCY LOSS

BY

© AMIE JANE CULLIMORE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled IMMUNOPEROXIDASE LOCALIZATION OF UREAPLASMA UREALYTICUM ANTIGEN IN CELL CULTURE AND IN TISSUES OF PREGNANCY LOSS submitted by AMIE JANE CULLIMORE in partial fulfilment of the requirements for the degree of Master of Science in Experimental Pathology in the Department of Pathology.

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Supervisor

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Date: October 6, 1986

This thesis is dedicated to my parents – with love.

ABSTRACT

The peroxidase-anti-peroxidase method was adapted to localize Ureaplasma urealyticum in tissues of spontaneous abortion. The technique was adapted first for use with smears of U. urealyticum and then for frozen sections of decidua which had been injected with the organism. The optimal concentrations of the primary antiserum, goat-anti-rabbit IgG and the PAP complex were determined using a checkerboard titration and were found to be 1:300, 1:100 and 1:100, respectively. After the method had been established in frozen sections, the technique was applied to paraffin-embedded tissues which had been injected with U. urealyticum.

The specificity of the primary antiserum was determined by absorbing with U. urealyticum, strain 27. The absorption was assessed using the modified metabolic inhibition test. The titre using the primary antiserum was 10,240 whereas the titre with the absorbed antiserum was <80, indicating that most of the antibodies against U. urealyticum had been removed. The specificity of the antiserum was further tested by incubating ureaplasma-infected monolayers with the absorbed antiserum using the immunofluorescence and immunoperoxidase methods. Negative results were also obtained when tissue was injected with M. hominis and incubated with anti-U. urealyticum antiserum using the immunoperoxidase assay. Finally, negative results were obtained when anti-M. hominis antiserum was used with the PAP method on tissues which had been injected with U. urealyticum.

After the establishment of the PAP technique in artificially injected tissue, paraffin-embedded spontaneously aborted tissues, which were naturally infected, were stained with the PAP method for ureaplasma antigen. Cases of spontaneous abortion were chosen on the following basis: the cases must have been positive in culture for ureaplasma and also have been detected after two days incubation as evidence that the organism was present in large numbers. In addition, there must have been some evidence of inflammation in the membranes, chorial plate and umbilical cord. Immunoperoxidase staining revealed that U. urealyticum could be identified at these levels.

A blind investigation of cases of spontaneous abortion and therapeutic abortion was used to determine the correlation between cultivation of the organism from fetal tissues and the localization of ureaplasma in the tissues with the immunoperoxidase assay. Complete agreement between these two techniques occured in 32 of the 52 (62%) tissues examined ($0.1 > p > 0.05$). The sensitivity of the culture method, with respect to the total number of positives using either method, was 78% and with the immunoperoxidase assay it was 66%. No relationship was found between the presence of inflammation in the tissues and the identification of the organism by either method.

The adaptation of the peroxidase-anti-peroxidase method for identifying U. urealyticum has made it possible to localize this organism in tissue. Thus, it may now be possible to elucidate the role of Ureaplasma urealyticum in reproductive pathology.

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LIST OF ABBREVIATIONS

PAP	Peroxidase-anti-Peroxidase
ml	millilitre
g	gram
min	minute(s)
µg	microgram
mg	milligram
sec	second(s)
r.p.m.	revolutions per minute
ul	microlitre
hr	hour(s)
R.T.	room temperature
v/v	volume per volume
w/v	weight per volume
LM	light microscope
CCU	colour change unit
µm	micrometre
DAB	diaminobenzidine tetrahydrochloride
MEM	Minimal Eagles Medium
mm	millimetre

CHAPTER ONE

LITERATURE REVIEW

I. INFECTION DURING PREGNANCY

Severe infection of the fetus may lead to fetal death, abortion or premature birth. When fetal infection is less severe, morphological malformations can result if infection occurs early in gestation or functional disorders if infection occurs later. Milder infection may have no effects at all (Plotkin, 1975).

This investigation was initiated to examine the role of Ureaplasma urealyticum in pregnancy loss. More specifically, the objectives of this study were to adapt the peroxidase-anti-peroxidase technique to localize this organism in routinely embedded fetal tissues and secondly, to investigate the relationship of inflammation in these tissues and the presence of U. urealyticum antigen within the tissues.

1.1 Infection and Perinatal Mortality

Two studies have reported a significant relationship between infection and perinatal mortality (Alberman, 1985; Buckell and Wood, 1985). Christensen (1982) found that 7 of 34 perinatal deaths were due to direct consequences of infection during pregnancy. Brans and colleagues (1984) used a larger population than Christensen and reported that,

although infection was not the most common cause of perinatal mortality, it was responsible for 19/320 of fetal deaths and 32/308 of neonatal deaths. The work of other investigators has not supported these findings. Royston and Geoghegan (1985) reported that in well nourished populations receiving good ante-natal care, intra-uterine infection has little if any part in the intra-uterine death after 28 weeks of gestation. Hovatta and associates (1983) supported these findings that infection is not a significant cause of stillbirth. Hovatta did not perform microbiological studies for a wide variety of organisms.

Blanc (1959) has proposed three ways in which amniotic infection can theoretically result. Firstly, an ascending infection could occur by cervical colonization. The surface of the endocervical epithelium is colonized and the microorganisms move up to the fetal membranes. At this point the microorganisms could enter the amniotic cavity and fluid and gain access to the fetus via transorificial spread, through the mouth, nose and Eustachian tubes. The second pathway by which microorganisms may invade is through hematogenous spread to the placenta and membranes with secondary invasion of the amniotic fluid. The third pathway of invasion is via infection down the fallopian tube.

Evaldson et. al. (1983) supported Blanc's hypothesis that amniotic infection may result from ascending infection. With immunofluorescence techniques, they showed that endocervical inoculation of Bacteroides fragilis, Streptococcus intermedius and Group B Streptococci all resulted in ascending infection in ewes. In all but one case, the ascending infection was arrested within the membranes and placentas of the ewes.

The pathology associated with complete amniotic infection is described at three levels (Blanc, 1959). Firstly, inflammation of the extra-placental membranes. This may include membranous deciduitis, chorionitis and chorioamnionitis. Secondly, inflammation of the chorial plate. A maternal and fetal response to infection may be seen at this level. Subchorionic intervillositis would imply a maternal response to infection. In some cases, the marginal decidua could be involved. Marginal deciduitis with an adjacent intervillositis could result. In the chorial plate, the fetus would respond by a vasculitis radiating towards the amnion if amniotic infection was present. The third level in which amniotic infection is manifested is in the umbilical cord. This is purely a fetal response. Vasculitis and inflammation of the Wharton's jelly, funisitis, may be evident. Guzick and Winn (1985) found that 61 of 244 preterm deliveries were associated with chorioamniotitis occuring alone or with the premature rupture of the membranes. They concluded that chorioamnionitis is a pathological marker for amniotic infection. Chellam and Ruston (1985) reported that approximately half of 200 liveborn and stillborn pregnancies had inflammatory lesions in the amnion and umbilical cord, the greatest incidence being associated with premature rupture of the membranes and placentas of the smallest and least mature infants.

Investigations of the ultrastructure of inflamed membranes have revealed extensive infiltration of chorion by neutrophils with phagocytic granules (Bartman and Blanc, 1970). Only in severely inflamed membranes were there focal areas of necrosis of amniotic epithelium with marked disruption of the amnion. In an in vitro investigation, Varner et. al. (1985)

infected amniotic epithelium and examined their ultrastructure. They noted progressive nuclear and basement membrane alterations.

1.2. Maternal Defense Mechanisms Against Infection.

The female genital tract possesses physical and biological mechanisms which aid in arresting microorganisms capable of establishing infection.

1.2.1. Physical Barriers

The distance between the site of infection and the placenta has a significant role in fetal colonization. Eschenbach (1985) found greater colonization of Group B Streptococci in the vagina than the cervix. As the distance increases between the site of infection and the placenta the number of organisms decreases and thus, so does the chance for fetal infection.

The progesterone dependent mucus plug also acts as a defense against potential pathogens. Bacterial movement is inhibited up the genital tract by the viscous plug (Blanc, 1959). Zuckerman and associates (1975) reported that cervical mucus has a strong inhibitory effect on many organisms. The cervical mucus was found to be inhibitory to all organisms tested. It had the greatest inhibitory effect of Micrococcus lysodeictus and the inhibitory strength on the other organisms tested were in the following order: S. albus, S. aureus, P. mirabilis, E. coli, C. albicans, S. haemolyticus and S. faecalis. The maximum inhibitory capacity was on the fourteenth day of the menstrual cycle whereupon it decreased.

Collagen content of the amniotic membranes may affect the occurence of premature rupture of the membranes (PROM) (Skinner et. al., 1981; Kanayama et. al., 1985). Skinner reported that collagen content in PROM was significantly lower than controls. These investigators also showed that collagen content decreases with gestation at 32 to 40 weeks. They suggested that premature weakening of the membranes, may be due to factors controlling synthesis and degradation of collagen.

1.2.1. Biological Barriers

Local inflammatory responses, namely leukocytic infiltration at levels of the cervical os and upper canal, all function to remove infecting microorganisms and, therefore, protect the fetus from contamination (Naeye and Ross, 1982). Intra-membranous (intra-amniotic) mechanisms exist which aid in halting microbial colonization of the fetus. Organisms gaining access to the amniotic fluid are diluted due to the relatively large volume of the amniotic fluid. As well, the large surface area of the amniotic cavity decreases the local effects of the microorganisms. This is termed membrane trapping. Organisms adhere to the membrane surface which are then targets for polymorphonuclear cells. In addition, the microorganisms migrate out into the maternal space, thus removing the pathogens from the vicinity of the fetus (Blanc, 1959).

Many investigators have reported antimicrobial effects of amniotic fluid. Thadepalli and colleagues in 1982 and Hemming et.al. in 1985 analyzed amniotic fluid for antimicrobial factors. They reported that amniotic fluid was inhibitory to E. coli if the phosphate/zinc ratio was less than 200 or if iron was less than 1.2 μg/ml and unbound transferrin

was greater than 40%. They also reported than the antimicrobial affects were increased as gestational age increased. Schlievert et. al. (1976) isolated a low molecular weight peptide which had antimicrobial activity and was phosphate sensitive. In a later study, Schlievert's group (1977) adjusted phospate/zinc ratios by varying zinc concentrations in amniotic fluids less than 20 weeks gestation. Amniotic fluid samples with phosphate/zinc ratios greater than 200 were non-inhibitory. Samples with a ratio between 100 and 200 were bacteriostatic and those samples with a phosphate/zinc ratio less than 100 were bactericidal. Feingold et.al. (1979) examined the possible relationship between antibacterial activity of amniotic fluid and its peroxidase content. They did not find peroxidase activity in any of the inhibitory samples. Larsen and colleagues in 1984 reported a low molecular weight fraction of human amniotic fluid was bactericidal for E. coli. They could not, however, offer any clues as to the nature of this factor. Larsen and Davis (1984) found that an increase in temperature would augment the inhibitory capacity of amniotic fluid.

Larsen et.al. (1983) found 13 of 32 amniotic fluids to be inhibitory. There was a difference in susceptibility of different strains of E. coli to the amniotic fluid inhibition. Feingold and colleagues (1979) reported antimicrobial activity to S. aureus in 50 of 76 amniotic fluid samples. Blanco et. al. (1982) examined the antimicrobial activity of amniotic fluid from women with intra-amniotic infection. Fifteen of 50 women with intra-amniotic infection had inhibitory amniotic fluid samples while 34 of 50 women without intra-amniotic infection had inhibitory samples. These investigators suggested that those women who are at risk of developing

intra-amniotic infection may have amniotic fluid which is not effective against invading microorganisms.

Maternal antibody has a significant role in halting microbial infiltration of the fetus. Two studies (Baker and Kasper, 1976; Wood et.al, 1984) have looked at the effect of maternal antibody on microorganisms. Baker and Kasper found that healthy neonates born to women with antibody to Group B Streptococci had antibody in umbilical cord serum suggesting transplacental transfer of immunoglobulins. Klegerman's group (1983) determined that a minimum concentration of maternal antibody was necessary for protection of mice against a 90% lethal dose of GBS. The protective antibody was primarily IgG in concentrations of 0.25-1.0 μg/ml. Antibody crossed the placenta and was present in the cord sera of infants of greater than 32 weeks of age.

2.3. Fetal Defense Mechanisms Against Infection

2.3.1. Physical Barriers

The fetus has physical barriers which also help to prevent pathogenic colonization. After twenty weeks of gestation the skin becomes keratinized and is impermeable to microorganisms. The umbilical cord has relatively impermeable stratified squamous epithelium. This is true of its entire length except for the small distal portion which is covered by columnar epithelium. The columnar epithelium is more permeable to invading organisms.

Intra-fetal vessels are protected by their depth within the fetal soma. Thus, in order to gain access to the fetal circulation,

microorganisms must enter the chorial plate or be aspirated or swallowed by the fetus and gain access via the viscera (Blanc, 1959).

2.3.2. Biological Barriers

Like the mother, the fetus also has biological defense mechanisms to combat microorganisms. In his 1985 review, Hill described the defenses which the neonate and fetus possess. Pre-B cells are found in the fetal liver as early as 8 weeks of gestation and immunoglobulins are found on the mature B cells at 10 weeks. By the fourth month, the number of immunoglobulin-bearing lymphocytes are near levels found in the adult. Complement components are synthesized early in fetal development (before twenty weeks of gestation). Levels are low until the third trimester but at term the hemolytic activity is half that of the adult. T lymphocytes are present in numbers that are equal to or greater than the adult.

2.4. Loss of Control at the Cervical Level.

In acute infection there is a great load. Local inflammatory responses may not be great enough to combat the infection and ascending infection may result (Naeye and Ross, 1982; Eschenbach, 1985). The loss of amniotic fluid and the effect on pregnancy outcome has been investigated by several groups. Taylor and Garite (1984) found 22 of 53 patients with premature rupture of membranes had amnionitis. Duration of the rupture of the membranes increased the risk of fetal infection (Naeye and Ross, 1982; Blanc, 1959). Taylor and Garite did not support these findings. Minkoff (1983) found an association of various vaginal organisms

with prematurity and premature rupture of the membranes. These findings have also been substantiated by Evaldson et. al. (1980). There was significantly higher colonization in patients with premature rupture of the membranes than in controls. Naeye (1982) investigated pre-term deliveries with premature rupture and the effects of coitus and chorioamnionitis. He found that in couples who continued coitus to term, infection was two to four times greater than those who did not. Naeye proposed that coitus with an infected partner may introduce bacteria into the vagina and thus, ascending infection may result. Seminal fluid has proteolytic enzymes which help facilitate the passage of sperm through the mucus plug and this may aid the passage of bacteria to the fetal membranes.

If the maternal response to infection is overwhelmed, fetal infection may result. If the load of organisms is too great, maternal polymorphs may not be able to remove all invading organisms from the decidua and the membranes. Infection may become more severe and spread to the fetus. Intra-amniotic spread may result if there is failure of dilution of the amniotic fluid due to premature rupture of the membranes (Blanc, 1959). If the antimicrobial factors of the amniotic fluid are ineffective, there is greater likelihood of fetal colonization (Naeye et. al., 1982). It has been reported that amniotic fluid from patients with intra-amniotic infection was less likely to be inhibitory to E. coli than patients free of infection (Blanco et. al, 1982; Gibbs et. al., 1982).

As already mentioned, the fetus does have an immune system which begins developing at an early stage. The fetal immune system is immature; it is deficient in IgM, IgA and some of the subclasses of IgG.

Opsonins to polysaccharride coated Gram positive and negative bacteria may be at low levels or absent. B lymphocytes are present but antibody production may be depressed or delayed compared to the adult. This is likely due to inadequate T and B cell or macrophage interaction. Complement components are decreased and the supply and function of polymorphonuclear cells are deficient. This may be exaggerated during stress. Hill (1985) stresses that the immature immune system of the fetus and neonate may underlie its propensity to develop intra-uterine infection.

2.5. Secondary Complications associated with Infection

2.5.1. Premature Labour

Bacteroides fragilis, Peptostreptococcus, Fusobacterium, several streptococci, E. coli, Klebsiella, Staph. epidermis, pneumococci, Lactobacillus and Mycoplasma hominis produce phospholipase A (Bejar et. al., 1981). Phospholipase A causes release of local arachidonic acid which stimulates prostaglandin formation. Prostaglandins are thought to cause myometrial activation by increasing gap junction formation. Thus, there is an increase in coordinated activity of the myometrium and labour results. (Bejar et. al., 1981). Phospholipase A has also been found to be released by U. urealyticum (Quinn et. al., 1986).

The occurence of fetal infection is dependent on many factors which have already been mentioned. The morbidity or mortality which may result is dependent on the dose of infection, age of the fetus and the

immune systems of the fetus and the mother. It is important to realize that cervical colonization does not necessarily correlate with fetal infection or death (Svensson et. al., 1986).

II. UREAPLASMA SPECIES

2.1. Diseases associated with Ureaplasma species

2.1.1. Respiratory Disease

Ureaplasma diversum (Howard and Gourlay, 1982) was reported by Howard et. al. (1976) to cause a cuffing pneumonia in gnotobiotic calves. The extent to which ureaplasmas play a role in respiratory disease in avian species is, at present, confused. Stipkovitis and Rashwan (1976) reported chickens to have air sacculitis and peritonitis. Koshimizu (1982) did not support these findings. Japanese fowl colonized with ureaplasmas did not have any associated lesions.

Conflicting evidence exists as to whether U. urealyticum causes respiratory distress in human neonates. Quinn et. al. (1983a) reported serological evidence implicating U. urealyticum as a cause of infection in the perinatal period. Eleven of 21 neonates with respiratory disease had titres of >1/32 compared to one of the 24 control neonates. These data were not supported by Taylor-Robinson and colleagues (1984), who found no significant difference in urea colonization of neonates with respiratory distress compared to controls.

2.1.1. Urethritis

U. urealyticum is known to cause non-gonococcal urethritis in humans and animals. In experimentally inoculated goats, ureaplasmas have caused a polymorphonuclear leucocyte response in the urethra (Taylor-Robinson et. al., 1979). There still exists a state of reluctance by some clinicians to regard ureaplasma as a causative agent of non-gonococcal urethritis (NGU) (Taylor-Robinson, 1984). This reluctance stems from the fact that ureaplasma can be isolated from healthy men as well as those with NGU. Taylor-Robinson offers the explanation that the existence of ureaplasma in the healthy male is likely due to its being present in sub-pathogenic numbers. Hunter and colleagues (1981) also support Taylor-Robinson's findings . Ureaplasma was present in significant numbers in patients with chlamydia negative non-gonococcal urethritis. Taylor-Robinson et. al. (1985) reported that ureaplasma is a cause of persistent urethritis in patients with hypogammaglobulinaemia.

2.1.3. Urinary Calculi

Experimental evidence exists supporting the idea that ureaplasma may be associated with urinary stone formation in rats. Friedlander and Braude (1974) found that inoculation of ureaplasma into rat kidney resulted in stone formation in 43 of the 69 rats. No stones were formed in any of the 17 controls. In examination of the urinary tract stones in humans, Petterson et. al. (1983) found 6/15 patients with stones of infectious nature, were colonized with U. urealyticum, in two patients the organism was in pure culture.

2.1.4. Vulvovaginitis and Cervicitis

Ureaplasma may be pathogenic in the lower genital tract of some animal species. Ball and McCaughey (1982) reported pronounced vulvitis in ewes after 2 to 3 days post-inoculation with ovine ureaplasmas. Similar results have been reported in cattle. Doig and colleagues (1980) after inoculating the uteri of Holstein cows, found 14/16 with vulvitis, 94% of which were culture positive in the first 4 days post-inoculation for ureaplasmas. Although there is strong evidence that ureaplasma causes vulvitis in certain animal species, there is no evidence that it may cause this lesion in humans. Moller's group (1985) found no significant difference in the presence of ureaplasma between women with cervicitis and women in the control population.

2.1.5. Abortion and Infertility

The role of this extra-cellular parasite in pregnancy loss and infertility in animals still remains controversial. Livingston and colleagues (1978) associated ureaplasma isolation with infertility in sheep. Their later work (1982) involved inoculating the ram of a breeding population of sheep and assessing pregnancy outcome. All ewes became infected after coitus. Fertility was greatly reduced compared to the control population. Birth weight was also reduced.

The evidence for U. urealyticum causing spontaneous pregnancy loss in humans still remains open to question. Embree et. al. (1980) found ureaplasma isolation of fetal membranes and placenta to be associated

with prematurity, lower birth weight and intra-uterine growth retardation. Studies by Kundsin and collegues (1984) also support Embree's conclusions. The presence of ureaplasma in the placenta suggested a transcervical migration from the lower genital tract. Two research groups (Shurin et. al., 1975 and Harrison et. al., 1973) associated ureaplasma isolation with adverse pregnancy outcome. Cultures were, however, obtained only from the cervix of the mother and not from the fetus or placenta. Shurin et. al. found that colonization did not correlate with placental inflammation. Tafari and associates (1976) found in 23 of 290 perinatal deaths, ureaplasma was responsible for congential pneumonia and death. In 71 couples with histories of pregnancy wastage, Quinn et. al., (1983c) found mothers and fetuses had responded immunologically to U. urealyticum. Sixty of the 71 couples were colonized with ureaplasma and 95% of these aborted. Cumming et. al. (1984) have described the phenomenon of mycoplasmal endometritis. These investigators correlated endometrial histology with the presence of ureaplamas in 11 of 32 patients. They have suggested that further work localizing the antigen in the tissue would support this histopathological feature.

Antibiotic therapy has been used to assess ureaplasmal involvement in spontaneous abortion (Quinn et. al., 1983b). Pregnancy loss was reduced to almost half in couples treated with doxycycline prior to conception compared to 22/24 in those not treated. Toth et. al. (1983) support Quinn's findings. Toth compared pregnancy rates in women in whose husbands infections had been eradicated. Pregnancies occurred in 77/129 couples whose ureaplasma infections had been eradicated compared

to 2 of 32 couples who still harboured the infection. Quinn suggested that U. urealyticum in the genital tract may have an important role in infertility. They are not sure, however, whether the difference in pregnancy rates is due solely to the eradication of mycoplasmas or the erradication of other potential pathogens along with the mycoplasmas in the genital tract. No strict internal controls were included and both studies by Toth and Quinn looked for the presence of mycoplasmas and not for any other potential pathogens of the female genital tract.

These findings are not supported by all investigators. Gump and associates (1984) found no relatonship between cervical ureaplasma infection and infertility. Cassell and colleagues (1983) did not isolate U. urealyticum more frequently in the endometrium of infertile patients than from fertile women during diagnostic laparoscopy. Thomsen and colleagues (1984) found no evidence that ureaplasma or Mycoplasma hominis could cross intact membranes suggesting that, in pregnancies where premature rupture of the membranes is not a threat, U. urealyticum may not be a major factor in pregnancy complications. These finding were contrary to the results found by Cassell's group (1983) although their population number (n=61) was smaller than Thomsen's (n=198).

2.1.6. Other Mycoplasma species associated with Reproductive Pathology

Mycoplasma hominis pathogenicity is primarily expressed in gynecological and obstetric infections. Under usual conditions, the

prevalence and number of Mycoplasma hominis is higher in the female genital tract than in the male or any other site in the body (Holmes, 1984). Although, Mycoplasma hominis has been associated with chorioamnionitis and funisitis, its clinical significance remains unclear (Embree et. al., 1984). Harwick and colleagues (1967) isolated Mycoplasma hominis from a fetus of a septic abortion. The mother had a 16-fold increase in titre to Mycoplasma hominis but not to other mycoplasma species tested (M. salivarium, M. orale, M. pneumoniae, M. fermentans). The placenta displayed nonspecific acute inflammation. Although bacterial culture of the cervical discharge also revealed moderate growth of Staphylococcus albus and Streptococcus fecalis, Mycoplasma hominis was the only microorganism isolated from the fetus.

Mycoplasma hominis has been isolated often with Ureaplasma urealyticum (Faur et. al., 1975; Alfa and Robertson, 1984). Mycoplasma hominis has been isolated from women with cervicitis or women whose sexual partners were men with NGU (Moller et. al., 1985). Experimental inoculation of the oviduct of female monkeys with Mycoplasma genitalium resulted in moderate to severe endosalpingitis. Histopathology revealed an acute inflammatory, luminal exudate with adhesions between the mucosal folds (Moller et. al., 1985).

Shurin and associates (1975) found no data to support the evidence that Mycoplasma hominis is associated with chorioamnionitis. Embree et. al. (1980) also found no significant difference in Mycoplasma hominis isolation between control placentas and those from high risk pregnancies.

2.1.7. Other Organisms Associated with Pregnancy Loss

Group B Streptococcal infection has been strongly associated in pregnancy complications. Group B streptococci are thought to adhere to vaginal epithelium (Anthony, 1982). Varner and colleagues (1985) demonstrated ultrastructural alterations of amniotic epithelium following incubation with Group B streptococci. Transmission electron micrographs showed progressive nuclear and basement membrane alterations. After experimental endocervical inoculation of ewes with group B streptococci, Evaldson and associates (1983) showed by immunofluorescence that group B streptococci were capable of establishing an ascending infection and might cause complications during pregnancy. Hoogkamp-Korstan et. al. (1982) demonstrated that neonatal aquistition of group B streptococci was dependent upon maternal carriage. In women who were permanently infected with group B streptococci, 26/41 neonates aquired the infection.

The mechanism by which group B streptococci evade the body's defenses is not completely understood. Skidmore and colleagues (1985) measured IgG levels to group B streptococci in maternal serum. Few women had antibody titres greater than 1:20 to GBS. This suggested that, by some means, group B streptococci may be evading the maternal immune system. Hemming's group (1985) demonstrated that group B streptococci could replicate in amniotic fluid samples that were bacteriostatic to E. coli.

Several viruses are also thought to have deleterious effects on pregnancy outcome. Although rare, rubella, cytomegalovirus and herpes

simplex II have been implicated in spontaneous abortion or severe congenital infection (Taina et. al., 1985).

Chlamydial infections in the genital tract have been associated with lower genital tract infections (Barnes et. al., 1985; Martin et. al. 1982) and salpingitis and pelvic inflammatory disease (Faro, 1985). Munday and colleagues (1984) found no evidence that Chlamydia trachomatis plays a role in spontaneous abortion. McKinlay et. al. (1985) however, reported two cases of severe Chlamydia psittaci sepsis during pregnancy. The infections appeared to be zoonotic, aquired from infected ewes. The placenta of one showed focal fibrin deposition and acute inflammation.

2.2. History of Ureaplasma urealyticum

In 1954 Shepard descibed tiny (T)-form pleuropneumonia-like organisms which had been isolated from patients with non-gonococcal urethritis. First known as T-Strain of Mycoplasmas, U. urealyticum colonies ranged from only 7 to 15 μm in diameter. Later, Shepard (1956) went on to describe its morphological characteristics in more detail. Still, analysis of this organism was limited because of the lack of information regarding its growth requirements. When it was discovered that U. urealyticum grew better at an acidic pH (Shepard and Lunceford, 1967) investigators were better able to cultivate the organism and obtain more knowledge about its isolation, identification, cultivation, biochemistry and serology.

With the identification of urease activity in the T-Strains (Shepard, 1966; Purcell et. al., 1966; Ford and MacDonald, 1967; Shepard

and Lunceford, 1967), a new genus and species was proposed; Ureaplasma urealyticum in the family Mycoplasmataceae (Shepard et. al., 1974).

2.3. Morphology of Ureaplasma urealyticum

2.3.1. Colonial Morphology

Shepard (1956) first described ureaplasma colonies as minute, irregular and spherical. Colonies are usually 15 to 60 μm in diameter (Taylor-Robinson and Gourlay, 1984) and with or without zones of surface growth. Colonial morphology is dependent upon growth conditions. Agar composition, gel strength, $MgSO_4$ concentration (Furness, 1973), manganese (Robertson and Chen, 1984) and gaseous conditions (Robertson, 1982) have been found to affect the growth of the organism.

2.3.2. Cellular Morphology

In liquid media, ureaplasmas are dense round organisms ranging in size from 0.3 to 0.8 μm in diameter (Shepard et. al. , 1974; Razin et. al., 1977) and are usually found singly or in pairs (Razin et. al., 1977; Robertson et. al., 1983). Their morphology is dependent on culture medium, pH, fixation and examination technique (Shepard, 1967).

2.3.3. Ultrastructural Morphology

U. urealyticum is bound by a single trilaminar membrane that is approximately 10 nm thick (Whitescarver and Furness, 1975). These investigators also reported an extramembranous hair-like structure, 5 to 8

nm long. The electron dense layer was also substantiated by Robertson and Smook (1976). Intra-cellular structures include 70S ribosomes and DNA (Razin, 1978). It is generally believed that mycoplasmas replicate via binary fission (Razin, 1978).

2.4. Nutritional Requirements and Biochemical Properties

Ureaplasmas are distinct from all other members of the mycoplasmas because of their ability to hydrolyze urea (Shepard and Lunceford, 1967). Other biochemical properties and nutritional requirement are outlined in Table 1.

2.5. Serological Differentiation of Ureaplasmas

Ureaplasmas from humans are antigenically distinct from ureaplasmas from other species and from other Mycoplasma species. Currently, 14 serovars exist in the species U. urealyticum. The progressive serotyping scheme of Stemke and Robertson (1985) is shown in Table 2.

Several methods have been used to serotype ureaplasmas. The metabolic inhibition test (Purcell et. al., 1966; Robertson and Stemke, 1979), the mycoplasmicidal test (Lin and Kass, 1970), the growth inhibition test (Black, 1970), colony epifluorescence (Clyde, 1967; Black, 1970; Stemke and Robertson, 1981), indirect immunoperoxidase (Polak-Vogelzang, 1978; Quinn et. al., 1981) and the enzyme-linked immunosorbent assay (ELISA) (Turunen et. al., 1982).

Table 1: Other Biological Properties of Ureaplasma urealyticum

Growth conditions, genome size and enzyme activity are presented. This table, modified from Taylor-Robinson and Gourlay (1984), is a summary of work by many investigators: (a) Robertson (1985); (b) Shepard and Lunceford (1965); (c) Black et. al. (1972); (d) Black et. al. (1972); (e) Rottem et. al. (1971); (f) Shepard and Lunceford (1967); (g) Vinther and Black (1974); (h) Delisle (1977); (i) Ajelli et. al. (1977); (j) Romano and LaLicatta (1978); (k) Black (1973); (l) Robertson et. al. (1984); (m) DeSilva and Quinn (1986); (n) Watanabe et. al. (1973); (o) Manchee and Taylor-Robinson (1970).

Property	Investigator
Growth optimal in 5% CO_2 and 95% N_2	a
Grow poorly under aerobic conditions	
Optimal pH 6.0 $\pm$ 0.5	b
Genome Size, daltons, 4.1-4.8 x 10^8	c
Mol % G + C of DNA, 26.9-30.2	d
Optimal temperature, 37°C	
Cholesterol required for growth	e
Enzyme Activity:	
Urease	f
a-Glycerophosphate dehydrogenase	h
Aminopeptidase	g
Esterase	h
L-Histidine ammonia-lyase	i
Malate dehydrogenase	h
Lactate dehydrogenase	h
Adenosine triphosphatase	j
Ribonuclease	j
Deoxyribonuclease	j
Phosphatase	k
IgA Protease	l
Phospholipase A and C	m
Proteolytic Activity	n
Hemolysis of erythrocytes	o
Hemadsorption of erythrocytes	o

Table 2: Expanded Serotyping Scheme of U. urealyticum

Serotype Number	Strain	Strain Identification	Scheme
1	7	Ford (1967)	Black (1970)
2	23		
3	27		
4	58		
5	354		
6	Pi		
7	Co		Robertson & Stemke (1982)
8	T960(C X8)	Shepard et. al.(1974)	
9	Vancouver	Ford & Smith (1974)	
10	Western	Robertson and Stemke (1979)	
11	K2	Lin et. al. (1972)	
12	U24		
13	U38		
14	U26		

(Stemke and Robertson, 1985)

The identification of different serotypes has led to the investigation of whether serotype specificity is associated with disease states. Shepard and Lunceford (1978) serotyped ureaplasmas from patients with NGU using the growth inhibition test. Serotype 4 was the most commonly found serotype in patients (52%) and controls (24%). Stemke and Robertson (1985) also found that serotypes 3 and 4 were among the most commonly isolated serotypes from patients with NGU. There was no difference, however, between the number of patients with serotypes 3 and 4 (33% and 13%) and the number of controls with these serotypes (37% and 15%). Robertson et. al. (1986) have looked at the serotypes of U. urealyticum isolated from spontaneous abortion and therapeutic abortions. Again, serotype 3 was most commonly found but no difference was found between the control population and patients, 29% and 31%, respectively.

III. ANTIGEN LOCALIZATION BY IMMUNOPEROXIDASE

3.1. Methods for the Detection of Antigen in Tissue

Immunoperoxidase techniques are powerful diagnostic tools which allow one to examine the specific immunological reaction of antibodies and histological morphology simultaneously. Direct and indirect immunoperoxidase methods involve the conjugation of horseradish peroxidase to the primary or secondary antibody, respectively. The unlabelled antibody method, the peroxidase-anti-peroxidase (PAP) method (Sternberger, 1970) and the biotin-avidin system (Guesdon et. al., 1979)

avoid the conjugation steps of the direct and indirect techniques. In the PAP assay, the sections are incubated with the primary antibody and then with a second antibody directed against the globulin fraction of the primary antibody. Following this step, a peroxidase-anti-peroxidase complex is added. The biotin-avidin system is based on the principle that the glycoprotein, avidin, has a high affinity for biotin. Avidin has four binding sites for biotin and thus, avidin-biotin complexes can be prepared.

Like the PAP technique, the glucose oxidase-anti-glucose oxidase (GAG) method involves an enzyme in the reaction. This method uses a microbial enzyme rather than horseradish peroxidase. It has been used, although not frequently, to visualize antigen in tissue (Clark et. al., 1982)

3.2 Sensitivity of the Immunotechniques

Several investigators have reported that the unlabelled immunoperoxidase techniques have a greater sensitivity than the labelled enzyme methods and immunofluorescence (Patrali et. al., 1974; Burns, 1975; Bosman, 1983). In contrast, results of other studies have indicated little difference in sensitivity (Muller-Hermelink and Hansman, 1984; Valnes et. al., 1984). Thus, it has become important to review the advantages and disadvantages of the respective techniques in order to decide which technique is best suited for the study.

3.3. Advantages and Disadvantages of the Immunotechniques

3.3.1. Direct and Indirect Immunofluorescence

Immunofluorescence techniques offer the advantage of being less time-consuming than other immunocytochemical techniques. A wide variety of conjugated antibodies are available commercially and rhodamine conjugates are also available, therefore, two antigens can be demonstrated simultaneously.

Immunofluorescence generally uses frozen, cryostat sections which can be limiting if retrospective studies are desired. Cryostat sections offer better antigen preservation, although histological features are often compromised. Sections stained by immunofluorescence can not be stored indefinitely as the fluorescent dyes are subject to fading with time. Examination of sections stained by immunofluorescence requires a special microscope which is costly and the process is tedious. Problems can arise during the labelling process, the antibody can be denatured and the fluorescent tag may become inactivated (Vandesande, 1979).

3.3.2. Direct and Indirect Immunoperoxidase Assays

Direct and indirect immunoperoxidase methods offer the advantage of less background, non-specific staining than immunofluorescence. Like immunofluorescence, they are less time consuming. Examination of the tissue is less tedious, as the light microscope is used. Sections stained by these methods are permanent and, therefore, storage can be indefinite.

Problems may arise during the conjugation process. The antibody may be denatured and the enzyme may be inactivated.

3.3.3. Unlabelled-Enzyme Immunocytochemical Techniques

The PAP technique is routinely used in surgical pathology (Falini and Clive, 1983). This method can be used with fresh frozen sections or with formalin fixed, paraffin sections, therefore, tissue morphology is preserved and retrospective studies are possible. Like the direct and indirect enzyme methods, the preparations are permanent and thus, slides may be stored indefinitely. This must be a practical consideration for some laboratories. The unlabelled enzyme methods avoid problems encountered during the labelling process. The GAG method and the PAP technique together, have been used to demonstrate two different antigens in the same tissue (Clark et. al., 1982).

The PAP method and the biotin-avidin immunoperoxidase assay have the problem of reacting with endogenous enzymes, peroxidase and biotin which can lead to false positive results. These problems can be overcome by pre-incubation with the enzyme's substrate. One disadvantage of the biotin-avidin methodology is the differences that exist in mutual affinity in the different preparations. Each new preparation should be tested (Falini and Clive, 1983).

3.4. Immunoperoxidase Assays to Localize Prokaryotes

Since the development of the peroxidase-anti-peroxidase technique by Sternberger et. al. (1970) to identify Treponema pallidum, many have reported localizing other prokaryotes with the various immunoperoxidase techniques. For example, Legionella pneumophilia was identified in pneumonic lung in a clinical case of legionella with the PAP technique (Boyd and McWilliams, 1982). McRill et. al. (1984) used the PAP assay to

identify various species of Salmonella from formalized cell suspensions, broth cultures of clinical isolates and in tissue suspensions from liver and spleen of infected mice. Indirect immunoperoxidase was used by Hsu et. al. (1979) to localize Bacteroides fragilis is tissue.

A variety of viral antigens have been identified with these immunocytochemical techniques. Abramowitz et. al. (1982) used indirect techniques to identify Cytomegalovirus mononucleosis antigen in lymph nodes and bone marrow aspirates. The PAP method has been used to study viral antigens in cell culture. Grover et. al. (1980) identified buffalopox virus in fibroblasts and Pearson et. al. (1979) used Vero cell to detect Herpes simplex antigen.

Small prokaryotic organisms, Chlamydiae, Rickettsiae and Mycoplasmataceae, have been localized with immunoperoxidase methods. Winkler et. al. (1984) used the PAP technique to identify Chlamydia trachomatis inclusions in the endometrium. In 1985, these investigators identified the same organisms using the biotin-avidin system. C. psittaci has been localized in tissue culture using direct immunoperoxidase methods (Finlayson et. al., 1985) and rickettsia with the indirect technique (Raoult et. al., 1985). The mycoplasmas have also been identifed with immunoperoxidase methods. Polak-Vogelzang et. al. (1978) used an indirect method to identify four species of Acholeplasma and 16 Mycoplasma species using unfixed colonies on agar blocks. The direct technique has been used to identify M. orale in a McCoy cell line (Chasey and Wood, 1984). M. pulmonis has been localized in paraffin embedded tissues and swabs of the upper respiratory tract using indirect

immunoperoxidase (Hill, 1978). As mentioned earlier, Quinn et. al. (1981) reported serotyping U. urealyticum by indirect immunoperoxidase using a modified method of Polak-Vogelzang on unfixed colonies on agar blocks.

CHAPTER TWO

MATERIALS AND METHODS

I. PROKARYOTIC ORGANISMS

1.1. Source of Genital Mycoplasma Strains

The strains of genital mycoplasma used were obtained from Dr. J.A. Robertson, Dept. of Medical Microbiology, University of Alberta, Edmonton, Canada who obtained U. urealyticum strains 7 (Serotype 1) and 27 (Serotype 3) from Dr. D.K. Ford, Dept. of Medicine, University of British Columbia, Vancouver, B.C. Strain T960(CX8) was obtained from Dr. M.C. Shepard, STD Control Laboratory, Occupational and Preventitive Medicine, Naval Regional Medical Center, Camp LeJeune, N.C., U.S.A. (Robertson and Stemke, 1982) and M. hominis strain 14027 from ATCC, Rockland, M.D..

1.2. Storage of Organisms

Organisms were stored as described by Robertson and Stemke (1982). Organisms in late logarithmic phase growth were stored after harvesting and suspension in 1 to 2 ml of saline at -70°C.

1.3. Cultivation of Organisms

1.3.1. Preparation of Media

Media for the propogation of U. urealyticum was prepared as described by Robertson (1978). Bromothymol blue broth consisted of 2.1 g of PPLO Broth without crystal violet (Difco Laboratories, Detroit, Michigan), 0.1 g yeast extract (Difco) and 1.0 ml of 0.4% bromothymol blue solution (Fisher Scientific Co., Fair Lawn, New Jersey) and 90.0 ml of glass distilled water. The basal medium was then sterilized for 20 min at 21 lb/in pressure. After cooling, the basal medium was supplemented with 10 ml pooled normal horse serum (Gibco) and urea (0.025% w/v) (Fisher Scientific) and 0.1 ml of 20 µg/ml glycyl-l-histidyl-l-lysine acetate salt (GHL) (Calbiochem, La Jolla, California). Urea and GHL were filter sterilized before supplementation. Ampicillin sodium (0.5 mg/ml) was added (Ayerst Laboratories, Montreal, Quebec). The pH of the broth was adjusted to 6.0 with 1N HCl and the medium was stored at -20°C.

1.3.2. Harvesting

Propagation of ureaplasma strains was carried out as previously described by Stemke and Robertson (1982). Cells removed from -70°C were sonicated (Sontegrator system 40, Fisher Scientific) for 10 sec prior to inocultation. A log phase culture was used to inoculate 3500 ml of B. broth with a final concentration of 1:10,000.

The inoculated broth was incubated at 37°C overnight until the medium reached a pH of between 6.9 to 7.1. At this time, the bottles of culture were immediately placed on ice. Cells were then collected by

centrifugation in a type 19 rotor in a Beckman L5-50 ultracentrifuge (Beckman Instruments Inc., Palo Alto, California) at 14,000 r.p.m. for 25 min. The supernatant was poured off without disruption of the pellet and the cells were resuspended in 0.85%(w/v) saline. The cell suspension was centrifuged in a Sorval RC 58 (DuPont Instruments, Newtown, C.T.) centrifuge for 25 min, 14,000 r.p.m. at 4°C. The supernatant was discarded and this pellet was resuspended in 1 to 2 ml sterile saline.

1.3.3. Determination of Colour Change Units$_{50}$

In order to determine the approximate number of viable cells in the harvested material, a 50% endpoint change was used as described by Reed and Muench (1938) and adapted by Ford (1972) into microtitre plates. The CCU_{50} per ml for each harvest which used 3500 ml of broth ranged between 3.0×10^7 to 1.0×10^8. The results were the mean of duplicate determinations.

1.3.4. Protein Assay

The protein content of the harvested organisms was determined by the Pierce BCA Assay (Pierce Chemical Co., Rockford,IL). A standard curve was made and the unknown protein concentration was determined. The protein concentration of the first antigen suspension was 1.93 mg/ml and 5.9 mg/ml for the second.

II. IMMUNOENZYMATIC TECHNIQUES

2.1. Immunological Reagents

2.1.1. Source of Immunological Reagents

Rabbit antiserum to strain 27, obtained from Dr. J.A. Robertson, University of Alberta, Edmonton, was raised against organisms cultivated in rabbit serum broth, according to Robertson and Stemke (1979). Goat-anti-rabbit IgG, rabbit peroxidase-anti-peroxidase complex and normal goat serum were all obtained from Bio/Can Scientific, Mississauga, Ontario. For immunofluorescence work, the fluorescein-isothiocynate conjugated goat-anti-rabbit IgG was obtained from Capell Biochemicals, Pennsylvannia.

2.1.2. Absorption of Primary Antiserum

In order to assess the specificity of the reagents, the anti-U. urealyticum antiserum was absorbed with its specific antigen. To 391 µl of phosphate buffer saline (Appendix), 1.25 µl of anti-U. urealyticum and 9.0 µl of U. urealyticum were added. This suspension was incubated for 2 hr at 37°C and then centrifuged in an Eppendorf 5414 centrifuge (Brinkmann Instruments, Westbury, NJ) at 4°C for 15 min at 12,000 r.p.m. The supernatant was decanted and stored at 4°C and served as the absorbed antiserum. The efficacy of the absorption was determined by the metabolic inhibition test (Sect. 2.1.3.) and by immunoperoxidase staining of ureaplasma-infected monolayers in which the primary antiserum was replaced by the absorbed antiserum (Sect. 3.4.). The antigen was prepared as described (Sect. 1.3.2.) and subjected to one freeze-thaw cycle. The final protein concentration was determined using the BCA protein assay

(Sect. 1.3.4.). The amount of antigen used to absorbed the antiserum was also increased to 45 µl and 90 µl.

2.1.3. Modified Metabolic Inhibition Test

A modified metabolic inhibition test (Robertson and Stemke, 1979) was used to determine whether the specific ureaplasma antiserum had been removed. To microtitre plates, 0.025 ml of B. broth was added to wells 2 to 12 of each horizontal row. The specific antisera were diluted in broth to 1:10 and 0.025 ml was added to wells 1 and 2 of each vertical row. Calibrated microdilutors (0.025 ml) were used to make doubling dilutions. The antigen, strain 27, was added to each well, in addition to 0.15 ml of 10% Guinea Pig complement (Hoeschst-Behring, France). The complement was diluted in broth and the pH was adjusted to 6.0 with 1.0N of HCl. In addition to the specific antiserum and its antigen, the absorbed antisera was used with strain 7 and T960(CX8). Controls included the antigens alone, the antiserum and broth. The microtitre plates were sealed with adhesive cellophane to prevent evaporation and vented. During incubation at 37°C, the plates were read twice daily until there was no change in colour for 24 hr.

2.2. Immunoperoxidase Assay on Smears of U. urealyticum

2.2.1. Titration of Primary Antiserum

Microscope slides were cleaned with acetone. From a 10^7 CCU per ml suspension of U. urealyticum, 0.1 ml was serially diluted in 0.9 ml of

sterile saline to titres of 10^4. Two loopful of each dilution were smeared onto each glass slide and allowed to dry.

The immunoperoxidase technique used was performed as described by Sternberger (1979). Slides were covered with a 1:25 dilution of normal goat serum and left in a humid chamber for 10 min. The slides were then blotted. Rabbit-anti-U. urealyticum was diluted from 1:20 to 1:2,000 in 0.9 ml of Tris-HCl. For one slide of each set, normal rabbit serum was substituted for the primary antiserum. Slides were incubated for 24 hr at 4^0C, washed with approximately 3 ml of Tris-HCl and then agitated in three changes of buffer for a total of 5 min. Goat-anti-rabbit IgG was added to the slides in a 1:100 dilution. The slides were put back into the humid chamber for 30 min at R.T. Slides were washed as described above and then blotted. The peroxidase-anti-peroxidase (PAP) complex in a 1:200 dilution was added for another 30 min at R.T. and slides were subsequently washed. To 0.05% (w/v) of diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, Mo.) diluted in Tris-HCl, 3% (v/v) hydrogen peroxide was added to a final concentration of 0.01% (v/v). The reaction was allowed to develop for 5 min. After washing, the slides were dehydrated in a series of ethanol baths (70%, 90% and 99%) and cleared in 2 washes of xylene for one min each. The slides were mounted in Permount (Fisher Scientific) and allowed to dry whereupon they were examined under the LM at 1000X.

2.2.2. Determination of Endpoint Staining

To determine the dilution of antiserum where staining ended, the primary antiserum was diluted to concentrations of 1:20 to 1:640,000. All slides contained about 3×10^3 CCU per ml. Controls included a slide with normal rabbit serum in place of the primary antiserum, a slide where the link reagent was replaced by Tris-HCl and finaly where the link reagent and PAP complex were replaced with buffer.

2.2.3. Cross-Reaction of Primary Antiserum with B. broth

Smears of U. urealyticum on glass slides were prepared as described (Sect. 2.2.1.). As well, a loopful of sterile B. broth was smeared onto a glass slide. Controls were as described above. To determine if endogenous peroxidase was present, slides were incubated with a 10%(v/v) solution of hydrogen peroxide in methanol.

2.2.4. Effect of Heat Fixation on Staining

One drop (0.025 ml) of 10^4 CCU per ml was spread onto a clean glass slide. After drying in air, half the smears were passed through a flame for fixation. Immunoperoxidase staining was carried out as described (Sect. 2.2.1.). The primary antiserum was used in a dilution of 1:1000 and 1:10,000. The controls were as described (Sect. 2.2.3.).

2.2.5. Effect of Incubation Time and Primary Antiserum Concentration

Staining of heat-fixed and air-dried smears was carried out as described (Sect. 2.2.1.). All slides were pre-incubated in 10% hydrogen

peroxide in methanol and then washed in three changes of fresh buffer. Incubation with the primary antiserum was for 30 min, 60 min and 24 hr.

2.3. Source and Injection of Sterile Decidua

2.3.1. Source of Tissue

Decidua from therapeutic abortions were obtained from Dr. L.H. Honoré, Dept. of Pathology, University of Alberta, Edmonton. All were checked for sterility in the following manner: Approximately 0.2 g of tissue was removed and placed in 2.0 ml of sterile saline. The decidua fragments were ground with sterile scissors in saline and 0.1 ml of each supernatant was serially diluted in 0.9 ml volumes of B. broth. The broths were incubated at 37°C. If no growth was present, as indicated by a colour change in the broth after 10 days, the broth was discarded. A loopful of the decidual supernatant was also streaked onto a blood agar plate and incubated at 37°C. The plates were discarded after 3 days if no growth was apparent.

2.3.2. Injection of Decidua with U. urealyticum

Using a 26 1/2 gauge needle, decidua fragments were injected with 0.1 ml of a 10^7 CCU per ml suspension of U. urealyticum, strain 27. Approximately 1.0 g of tissue was injected in three to four random sites and then was embedded in OCT embedding compound (Miles Laboratories, Naperville, IL). The tissue was cooled to -20°C for frozen sections.

If paraffin sections were required, the injected tissue was placed in 10% buffered formalin (Fisher Scientific) and processed by the Dept. of

Pathology, University of Alberta Hospitals, Edmonton. The fixation period was approximately 24 hr whereupon dehydration took place. An automated tissue processor (Technicon, Calgary, Alberta) was used. Tissue samples were transfered to 2 changes of 70% ethanol for 40 min each, then to 95% ethanol for another 40 min. The tissue was automatically transfered to 2 changes of absolute ethanol for 30 min and 50 min before clearing in xylene for 30 and 40 min. Following clearing, the tissue was infiltrated with paraffin. All reagents were supplied by BDH Chemical Co., Edmonton, Alberta.

2.4. Immunoperoxidase on Frozen Sections

Unfixed tissue was embedded as described above. After the embedding media had hardened, 5 μm sections were cut at -20°C on a cryostat (American Optical Scientific Instruments, Buffalo, N.Y.). The sections were picked up on glass slides and stored at -20°C until staining.

2.4.1. Indentification of Background Staining

Immunoperoxidase staining was carried out as described (Sect. 2.2.1.). The primary antiserum was used in dilutions of 1:100, 1:1000, 1:10,000. Each set of slides included a normal rabbit serum control, a non-infected tissue section which was pre-incubated with 10% hydrogen peroxide in methanol or water and non-infected tissue which was not subjected to the pre-incubation step. After staining, the slides were counter-stained in Harris' Hematoxylin (Appendix).

2.4.2. Variation of Incubation Time with Primary Antiserum

Infected decidua was stained by immunoperoxidase and the primary antiserum was used in 10-fold dilutions ranging from 1:100 to 1:10,000. Slides were incubated with the primary antiserum for 60 min and 24 hr at 4°C. Staining was assessed in terms of the intensity of the specific staining in relation to the amount of non-specific background staining.

2.4.3. Titration of the Link Reagent and PAP Complex

To determine the optimal staining concentrations, the primary antiserum, link reagent and the PAP complex were titrated in a checkerboard pattern.

Table 3: Titration of the Immunological Reagents

Reagent	Dilution
Primary Antiserum	1:300, 1:500
Link Reagent	1:50, 1:100, 1:200
PAP Complex	1:100, 1:200, 1:400

2.5. Staining with Paraffin Embedded Tissue

2.5.1. Preparation of Tissue for Staining

Decidua found to be free of microorganisms was obtained and injected as described (Sect. 2.3.2.) Paraffin blocks were pre-cooled in a -10°C cold tray (AO Scientific) for 15 min prior to sectioning. Four to five μm sections were cut from the blocks on a rotating microtome (AO Scientific). The ribbon of sections was then floated on a 50°C water

bath. To the heated water, approximately 1.0 g of gelatin was added to ensure adhesion of the section to the slide. After separation, the individual section was picked up onto a clean glass microscope slide and placed for 20 min in a 70°C circulating air dryer (Lipshaw Mfg., Detroit, Mich.). Slides were then stored at R.T. until staining.

2.5.2. Staining Procedures for Paraffin Embedded Tissues

In preparation for staining, sections were de-paraffinized through the following series of xylene and ethanol baths: Slides were immersed for 3 min each in 2 changes of xylene and then brought to water by a graded alcohol wash (absolute, 90% and 70% ethanol). Finally, the slides were immersed in distilled water for 5 min. The staining procedure for all tissues embedded in paraffin was as follows: To block endogenous peroxidase activity, the sections were incubated in 10% hydrogen peroxide in methanol for 30 min on a rotator (Fisher Scientific) (100 r.p.m.). Tissues were washed for 5 min in Tris-HCl. All washing was carried out with constant agitation. Non-specific protein sites were blocked with a 1:25 dilution of normal goat serum for 10 min whereupon they were blotted and rabbit-anti-U. urealyticum was added. All incubations took place in a humid chamber. The primary antiserum remained on the sections for 24 hr at 4°C. Following this incubation, tissues were rinsed with approximately 3 ml of Tris-HCl and then washed for 5 min with 3 changes of buffer. After blotting the sections, the link reagent, goat-anti-rabbit IgG was added to the sections for 30 min at R.T. after

which they were washed. Similarly, the PAP complex was placed on the slides in the humid chamber and incubated for 30 min at R.T.

For visualization of the reaction, 0.05%(w/v) diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl with 0.01%(v/v) hydrogen peroxide was added. The DAB had previously been suspended in Tris-HCl at the desired concentration and stored at -20°C. DAB was thawed and filtered prior to use and then 33 µl of a fresh 3% solution of hydrogen peroxide was added. After 5 min, the slides were washed for 5 min in running water. Counter-staining was achieved by immersing the sections in filtered Harris's Hematoxylin diluted 1:1 in distilled water for 10 to 15 sec. Excess stain was removed by washing the slides in running tap water for 5 min. After dehydration in graded ethanol baths (70%, 90% and absolute), sections were cleared in two changes of xylene and mounted with Permount.

In all experiments, controls included normal rabbit serum in place of the primary antiserum and a section which was not injected with U. urealyticum. When the primary antiserum had been absorbed with U. urealyticum, this absorbed antiserum was also included as a control. The slides were graded on the intensity of the specific staining, the number of positive fields at 40X and the amount of relative background, non-specific staining.

2.5.3. Titration and Incubation Time with the Primary Antiserum

To sections of decidua pre-injected with U. urealyticum, the primary antiserum was diluted to concentrations of 1:50 to 1:2000 and

stained (Sect. 2.5.2.). Incubation times with the primary antiserum were 24 hr and 48 hr.

2.5.4. Determination of Specificity of the Primary Antiserum

Sections containing U. urealyticum were prepared for staining as described (Sect. 2.5.1.). In addition to routine controls, rabbit-anti-M. hominis antiserum was included to assess whether any cross-reaction would occur with an antiserum to a different mycoplasma species.

Similarily, M. hominis (strain 14027) was cultured and harvested as described (Sect. 1.3.2.). Decidua was injected (Sect. 2.5.1.). Four to five µm sections were cut and stained. Using anti-U. urealyticum antiserum, sections containing M. hominis were stained with routine controls and sections containing U. urealyticum. Cross-reactivity was assessed under the LM.

2.6. Immunoperoxidase Staining on Tissues of Pregnancy Wastage

2.6.1. Staining of Cases Known to Harbour U. urealyticum

Five cases of pregnancy wastage, 4 spontaneous abortions and one still birth, were chosen for immunoperoxidase staining. The criteria for selection of the cases was as follows: Firstly, the case must have been U. urealyticum positive in culture in most tissues. The organism must have been isolated rapidly from the tissue, as indicated by a rapid colour change in broth. This was evidence that the organism was present in large numbers. Secondly, there must have been some evidence of inflammation in the placenta, decidua and/or fetal membranes. In addition to the five

cases infected with U. urealyticum, one case was chosen which was culture positive for M. hominis to assess the amount of cross-reactivity with the primary antiserum. The microbiological and pathological data were obtained from Dr. J.A. Robertson and Dr. L.H. Honoré, respectively. Staining was carried out as described (Sect. 2.5.2.). The staining was graded upon intensity, ranging from +++ (intense), ++ (moderate), + (mild) to - (no staining).

2.6.2. Blind Investigation of Tissues for U. urealyticum Antigen

A one-way blind study of 16 cases of pregnancy wastage was set up to investigate the correlation between culture positive cases and immunoperoxidase positive cases. Fifty-two tissues were chosen based on the following criteria: All cases were either late spontaneous abortion or late therapeutic abortion ranging in gestation age from 16 to 20 weeks. Twenty-four tissues were chosen because they were negative in culture for Ureaplasma and 28 were positive. The tissues were selected by Dr. L.H. Honoré, Dept. of Pathology, University of Alberta, Edmonton. The investigator reading the immunoperoxidase slides had no knowledge of the microbiological results. Positivity was based on intensity of staining which ranged from very intense (+++), moderate (++), weak (+) to negative (-). The number of positive fields at 40X, was arbitrarily recorded as +++ (>5 fields), ++ (3-5 fields), + (1-2) fields and - (no staining).

III. Eukaryotic Cell Cultures

3.1. Maintenance of Cell Culture

HeLa 229 cells were removed from storage in liquid nitrogen and immediately placed in a 37°C water bath. After thawing, the cells were resuspended in MEM medium (Appendix) to 10 ml and incubated at 37°C. When a confluent monolayer was formed, the cells were subcultured as follows: The medium was removed and the monolayer was rinsed with sterile PBS (pH 7.0). The buffer was poured off and 1.5 ml of 0.25%(w/v) trypsin (Difco Laboratories) were added. The tissue culture flask was gently rocked for 30 sec and the excess trypsin was poured off. The flask was incubated at 37°C until the cells had detached, about 4 min. The surface of the flask was rinsed with 10 ml of MEM and the cells in the suspension were dispersed by expelling them several times with a pipette. One-third of the trypsinized monolayer was transfered to each new flask (Corning Glass Works, Corning, N.Y.) and 6 ml of medium were added.

3.2. Preparation of Monolayer for Attachment of U. urealyticum

Attachment of the ureaplasmas to eukaryotic cells was assayed as described by Alfa (1986). HeLa cells were grown on 12 mm coverslips which had been acid-washed and stored in 95% ethanol. The coverslips were flamed and put into sterile 13 mm shell vials. Then 6×10^4 cells were inoculated into each shell vial. These produced a confluent monolayer within 24 hrs. HeLa cell counts were based on hemocytometer readings. Before attachment studies were completed, confluency was assessed by examination of the monolayer under the inverted microscope.

3.3. Attachment of U. urealyticum to the Monolayer

Ureaplasmas were cultured in B. broth until a pH of between 6.9 to 7.1. The cultures were then harvested as described (Sect. 1.3.1.). The final pellet was resuspended in H buffer (Appendix) and 0.2 ml was dispensed into each shell vial. The titre of U. urealyticum in the vial was approximately 1 x 10^8 CCU_{50} per ml. The vials were stoppered and incubated at 4°C for one hour.

3.4. Immunofluorescence on the Monolayer

Immunofluorescence was carried out as described by Alfa (1986). The supernatant fluid was aspirated from the shell vials and the monolayers were rinsed twice with H buffer. The coverslips were fixed for 10 min in acetone and rinsed again with H buffer. Two hundred µl of a 1:100 dilution of rabbit-anti-U. urealyticum serum was added to each vial. Controls included normal rabbit serum and absorbed rabbit serum. The vials were stoppered and incubated at 37°C for 15 min. The antiserum was then aspirated and washed four times with H buffer. Fluorescein-isothiocynate conjugated goat-anti-rabbit IgG was added to each shell vial for 15 min at 36°C. The antiserum was aspirated and washed. The last wash was left on overnight at 4°C. Coverslips were inverted onto glass slides with a drop of mounting fluid (10% glycerol in H buffer) and viewed under the fluorescent microscope at 40X.

3.5. Immunoperoxidase on the Monolayer

Confluent monolayers were infected as described (Sect. 3.3.). The monolayers were fixed for 10 min in acetone after which they were washed in Tris-HCl. Endogenous peroxidase was blocked by incubation with 10% hydrogen peroxide in methanol for 30 min. After washing for 5 min in three changes of buffer, non-specific protein sites were blocked with normal goat serum (1:25) for 10 min. Anti-U. urealyticum antiserum (0.025 ml) was added to the monolayers for 15 min at 37°C. Controls included anti-M. hominis antiserum in place of anti-U. urealyticum, normal rabbit serum, absorbed antiserum and a monlayer which was not infected with U. urealyticum. The coverslips were washed and then incubated with 0.025 ml of the link reagent for 30 min. Following washing, the coverslips were exposed to the PAP complex for 30 min at R.T. DAB was added, after washing for five min. The coverslips were dehydrated and cleared in ethanol and xylene and mounted onto glass slides with Permount.

CHAPTER THREE

RESULTS

1.1. Immunoperoxidase Staining using Smears of U. urealyticum

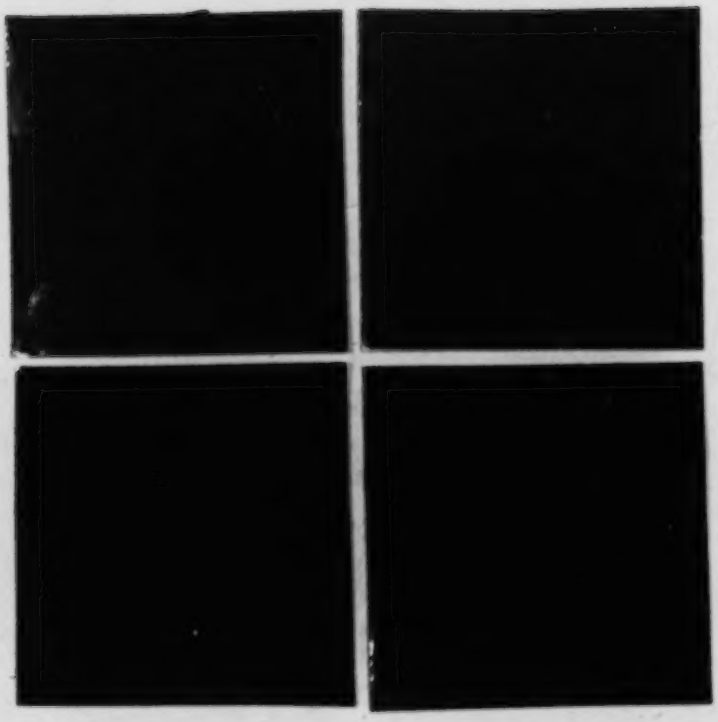
1.1.1. Titration of the Primary Antiserum

Smears of U. urealyticum were prepared on clean glass slides and stained (Ch.II.,Sect. 2.2.1.,pg.34). The intensity of the immunoperoxidase staining was compared in two ways: Firstly, among the differing concentrations of organisms and, secondly, for each dilution of antigen that was streaked onto a slide the primary antiserum was diluted to different concentrations. Therefore, the intensity with the primary antiserum concentrations was compared within each titre of organism used. At concentrations of 10^7 organisms, it was difficult to distinguish between the organisms and other small particles in the harvest suspension. At titres of 10^6 and 10^5, brown spheres were seen under oil immersion but there was no apparent size uniformity. The optimal concentration of antigen was 10^4 CCU (Fig. 1). Brown spheres were apparent and their size was more uniform than when less antigen was present.

The initial titration of the primary antiserum, anti-U. urealyticum, revealed that optimal staining was somewhere between a 1:200 and 1:2000 dilution (Fig. 1c). Endpoint staining was acheived at 1:640,000 (Fig 1d).

Figure 1: Immunoperoxidase Staining on Smears of U. urealyticum

Smears of U. urealyticum were streaked onto clean glass slides (Ch. II., Sect. 2.2.1., pg. 31). The normal rabbit serum control (a) exhibited staining of particles similar to that of the smears incubated with anti-U. urealyticum antiserum in a concentration of 1:1000 (c) (arrows). The slide incubated with diaminobenzidine only (b) showed a few pale staining particles. Staining of the smears decreased greatly when the antiserum was diluted to 1:640,000 (d). X1600. The bar represents 10 μm.



The normal rabbit serum control showed a significant amount of non-specific staining (Fig. 1a). The stained areas could have been mistaken for ureaplasmas. However, comparison of the staining with normal rabbit serum and the specific antibody showed that the latter had a greater staining intensity.

1.1.2. Non-specific Staining on Smears

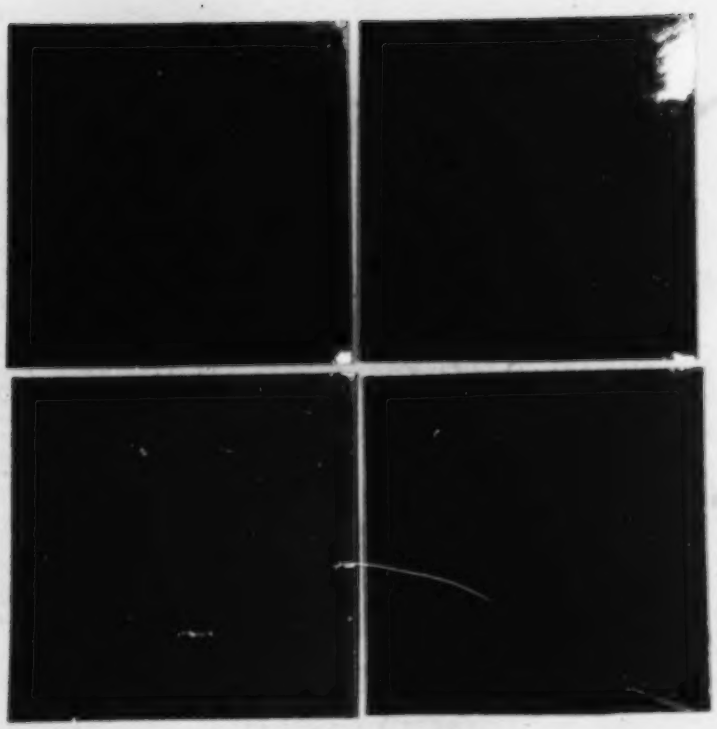
Since ureaplasmas are grown in B. broth and as B. broth contains large amounts of protein, it is possible that background staining may result from either antibody in the primary antiserum against certain broth components, such as yeast precipitates, or non-specific staining of the primary antibody to these components (Figure 2). Smears of U. urealyticum and smears of B. broth were prepared and stained (Ch. II., Sect. 2.2.3., pg.36) to determine if any cross-reactions were occurring. Background staining was apparent in the controls as well as smears of U. urealyticum. The smears of B. broth showed pale brown staining particles (Fig. 2b). When the smears of U. urealyticum were pre-incubated with hydrogen peroxide in methanol, the background staining was not reduced.

1.1.3. Heat Fixation of Smears

Smears of U. urealyticum were prepared (Ch. II., Sect. 2.2.1., pg.34). The smears were then heat-fixed (Ch.II., Sect. 2.2.3., pg.36) and stained (Ch. II., Sect. 2.2.1., pg.34). The process of fixing the smears of U. urealyticum did not affect the staining significantly. There was a slight reduction of non-specific staining in the heat fixed smears but the specific staining also seemed to be reduced.

Figure 2: Non-Specific Staining of U. urealyticum Smears and B. broth

Smears of U. urealyticum were prepared as described (Ch. II., Sect. 2.2.3., pg. 36). In addition, smears of B. broth were prepared to assess the amount of background, non-specific staining. Staining with normal rabbit serum (a) showed some particles (arrows). Immunoperoxidase staining of broth (b) revealed pale background staining particles (arrows). Pre-incubation of U. urealyticum smears with hydrogen peroxide (d) did not reduce background staining considerably when compared to smears which were not subjected to the pre-incubation step (c). X1600. The bar represents 10 μm.



1.1.4. Variation of Incubation Time with the Primary Antiserum

The incubation time of the smears with the primary antiserum was varied from 30 min, 60 min to 24 hr (Ch II., Sect. 2.2.5., pg.36). There was very pale staining at 30 and 60 min in smears which were heat fixed and those which were not. The staining of smears was much more intense at 24 hr for both the heat-fixed and non-fixed smears.

2.1. Immunoperoxidase Staining of Frozen Sections

2.1.1. Non-Specific Staining of Non-Injected Tissue

Sections were made from tissue in which no ureaplasmas were injected. When these were incubated with normal rabbit serum, background staining resulted (Fig. 3a). The tissue which had been incubated with the specific antiserum for U. urealyticum but not pre-incubated with hydrogen peroxide in methanol, showed background staining which was more intense than the normal rabbit serum (Fig 3b). When sections were pre-incubated in hydrogen peroxide in water, severe disruption of the tissue morphology made assessment of the staining difficult (Fig. 4b). However, replacement of the water component by methanol allowed better morphological preservation.

2.1.2. Variation of Incubation Time

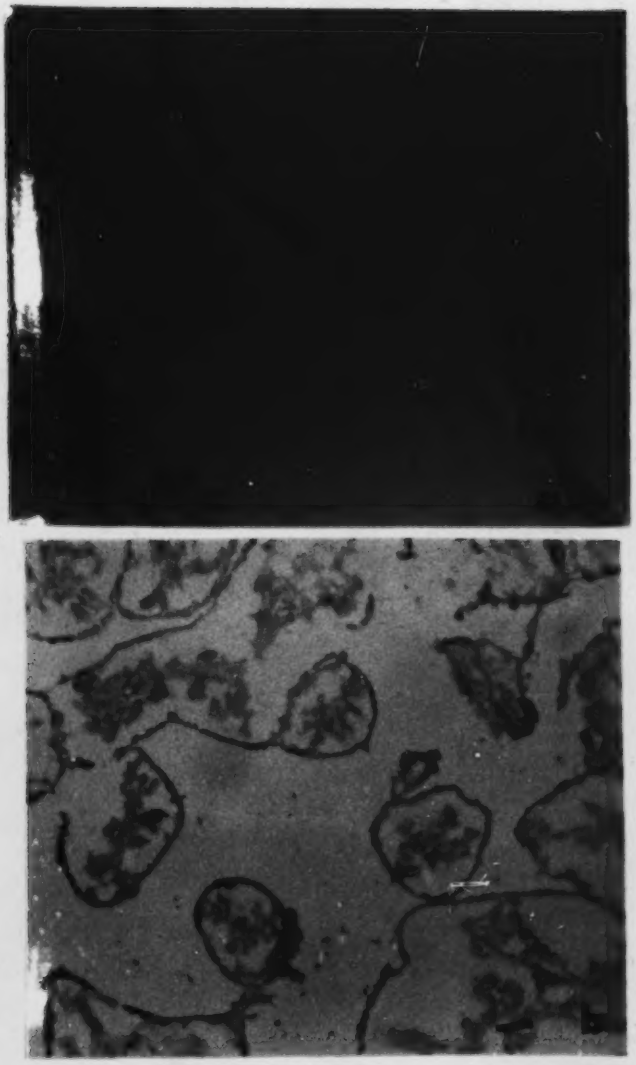
Frozen sections of U. urealyticum injected decidua were stained (Ch. II., Sect. 2.4.2., pg.38). The concentration of the primary antiserum was varied from 1:100 to 1:10,000 and the incubation time of the primary antibody was varied for 60 min and 24 hr. Examination under the LM revealed high background staining at a concentration of 1:100 for both

Figure 3: Non-Specific Staining Present in Sterile Tissue

Frozen sections of sterile maternal decidua were stained with the immunoperoxidase assay (Ch. II., Sect. 2.4.1., pg. 38). Pale brown staining was apparent in sections incubated with the normal rabbit serum in place of the primary antiserum (a). Background staining was also apparent in sections which had been incubated with the primary antiserum (b). X640. The bar represents 10 μm.

Figure 4: Non-Specific Staining in Sterile Tissue

Frozen sections were prepared as described in Figure 3. Pre-incubation of the sections with hydrogen peroxide in methanol (a) decreased background staining. When distilled water was substituted for the methanol, severe disruption of histological morphology resulted (b). (a) X640; (b) X320. The bar represents 10 μm.



times. No staining was apparent at primary antiserum concentrations of 1:5000 and 1:10,000 (Table 4). Normal rabbit serum controls were negative for both incubation times (Fig. 5 a & b). The optimal concentration of the primary antiserum was 1:500 for 24 hr (Fig. 6b).

2.1.3. Titration of Link Reagent and PAP Complex

Frozen sections of decidua containing U. urealyticum were stained and the concentrations of the immunoreagents were varied (Ch. II., Sect. 2.4.3., pg.39). The optimal concentration of the link reagent and the PAP complex was determined using concentration of the link reagent at 1:50, 1:100, 1:200. The PAP complex was used in concentrations of 1:100, 1:200 and 1:400. The primary antiserum was used in concentrations of 1:300 and 1:500. Staining intensity varied from no staining (-), weak staining (+) to moderate (++) and intense staining (+++). The optimal concentrations of the immunoreagents were 1:300, 1:100 and 1:100 for the primary antiserum, link reagent and the PAP complex, respectively (Table 5).

3.1. Immunoperoxidase Staining on Paraffin Embedded Tissue

3.1.1. Titration of the Primary Antiserum

Paraffin embedded tissue sections injected with U. urealyticum were stained (Ch. II., Sect. 2.5.2., pg.40). The primary antiserum was diluted from 1:50 to 1:2000. The incubation time with the primary antiserum was increased from 24 hr to 48 hr (Ch. II., Sect. 2.5.3., pg.41). Examination under the LM revealed background staining at concentrations

Table 4: Variation of Incubation Time and Primary Antiserum Concentration in Frozen Sections

Antiserum Concentration	Incubation Time	
	60 min	24 hr
1:100	*	*
1:500	++	+++
1:1000	+	++
1:2000	+	+
1:5000	-	-
1:10,000	-	-

* high background, +++ intense, ++ moderate, + weak, - no staining

Table 5: Titration of Link Reagent and PAP Complex

Primary Antiserum Concentration	Link Reagent	PAP Complex	Intensity
1:300	1:50	1:100	+
		1:200	+
		1:400	-
	1:100	1:100	+++
		1:200	+
		1:400	+
	1:200	1:100	+
		1:200	+
		1:400	-
1:500	1:50	1:100	+
		1:200	++
		1:400	++
	1:100	1:100	+
		1:200	++
		1:400	-
	1:200	1:100	-
		1:200	-
		1:400	-

- no staining, + weak, ++ moderate, +++ intense

Figure 5: Variation of Incubation Time and Primary Antiserum Concentration

Decidua was injected with U. urealyticum. Four um frozen sections were taken and sections were stained with immunoperoxidase. Negative controls of normal rabbit serum were negative at 60 mins (a) and 24 hrs (b). X640. The bar represents 10 μm.

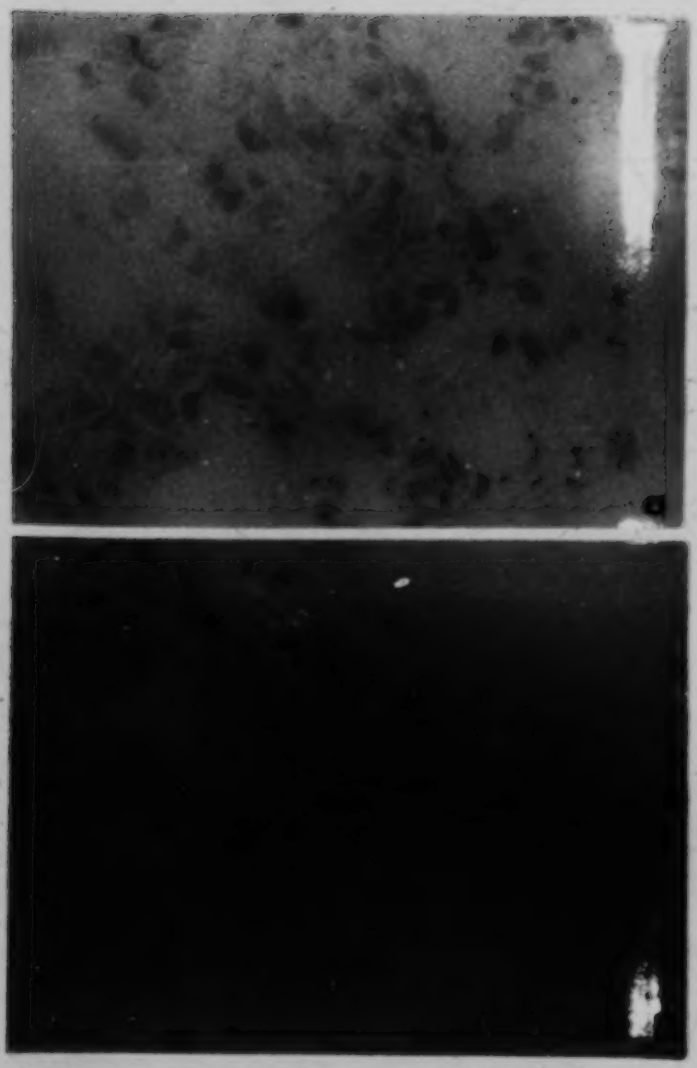


Figure 6: Variation of Incubation Time and Primary Antiserum Concentration

Frozen sections of decidua which had been injected with U. urealyticum were stained (Ch. II., Sect. 2.2.1., pg.34). The primary antiserum concentration was varied from 1:100 to 1:10,000 with incubation times of 60 mins and 24 hrs. Pale brown staining was apparent in sections which were incubated with a 1:500 dilution of antiserum for 60 mins (a). The intensity of the staining, with the antiserum at a dilution of 1:500, greatly increased when an incubation time of 24 hrs was used (b). Dark brown precipitate was evident in these sections (arrows). X640. The bar represents 10 μm.

of 1:50 and 1:100 (Table 6). The maximum staining intensity with low background staining was observed at an antiserum concentration of 1:400 for 24 hr (Fig. 8). Since there was no difference in staining between the two incubation periods, the more convenient incubation time of 24 hr was used in subsequent studies.

3.1.2. Specificity of the Primary Antiserum

To assess if the anti-U. urealyticum antiserum was specific for its antigen, antiserum to another mycoplasma, anti-M. hominis, was used in place of the ureaplasma antiserum (Ch. II., Sect. 2.5.4., pg.41). The normal rabbit serum control was negative as was the section which had not been injected with U. urealyticum. The positive control, a section containing ureaplasma and incubated with the specific antiserum, was positive (Fig. 10). The sections which had been incubated with anti-M. hominis were negative for any dark brown precipitate (Fig. 9b).

The specificity of the primary antiserum was further tested by injecting tissue with M. hominis and staining the sections with ureaplasma antiserum (Ch. II., Sect. 2.5.4., pg.41). The normal rabbit serum control and the non-injected tissue section were both negative. The positive control, described above, was positive. The tissue which had been injected with M. hominis was however, negative (Figs. 10a).

4.1. Absorption of the Primary Antiserum

The antigen was harvested and used within a day to absorbed the antiserum. The absorption was carried out as described (Ch. II., Sect.2.1.2.,pg.33). The metabolic inhibition test (Ch. II.,Sect.2.1.3., pg.34).

Table 6: Titration of Primary Antiserum for Paraffin Sections

Primary Antibody Concentration	Staining Pattern	
	24 hr	48 hr
1:50	*	*
1:100	+	+
1:200	+	+
1:400	+++	+++
1:800	++	++
1:1000	+	+
1:2000	+	+

* high background, +++ intense, ++ moderate, + weak, - no staining

Figure 7: Titration of Primary Antiserum with Paraffin Embedded Tissue

U. urealyticum injected decidua was paraffin embedded and 4 um sections were taken (Ch. II., Sect. 2.5.1., pg.39). The primary antiserum concentration was varied from 1:50 to 1:2000 for 24 hrs and 48 hrs (Ch. II., Sect. 2.5.3., pg.41). The normal rabbit serum control, where the normal rabbit serum was substituted for the primary antiserum, (a) was negative. When the anti-U. urealyticum antiserum was used in a dilution of 1:100 (b), high background staining was obvious. Background staining decreased when the primary antiserum was used in higher concentrations. This is obvious in the micrographs which follow. X640. The bar represents 10 μm.

Figure 8: Titration of the Primary Antiserum in Paraffin Embedded Tissues Injected with U. urealyticum

Tissue injected with U. urealyticum was paraffin embedded and 4 um section were made. The sections were stained using different concentrations of the primary antiserum (Ch. II., Sect. 2.5.3., pg.41). Sections incubated with incubated with the primary antiserum at dilution of 1:400 for 24 hrs (a) revealed markedly reduced background staining. Staining was still apparent but reduced in intensity, when the antiserum was used in a dilution of 1:2000 for 24 hrs (b). X640. The bar represents 10 µm.

Figure 9: Specificity of the Primary Antiserum

The specificity of the primary antiserum, anti-*U. urealyticum* was tested firstly by incubating tissue section which had been injected with *M. hominis* (a). Secondly, specificity was assessed by incubating *U. urealyticum* injected tissue sections with antiserum raised against another *Mycoplasma* species, namely, *M. hominis* (b). Both resulted in negative staining patterns. X640. The bar represents 10 μm.

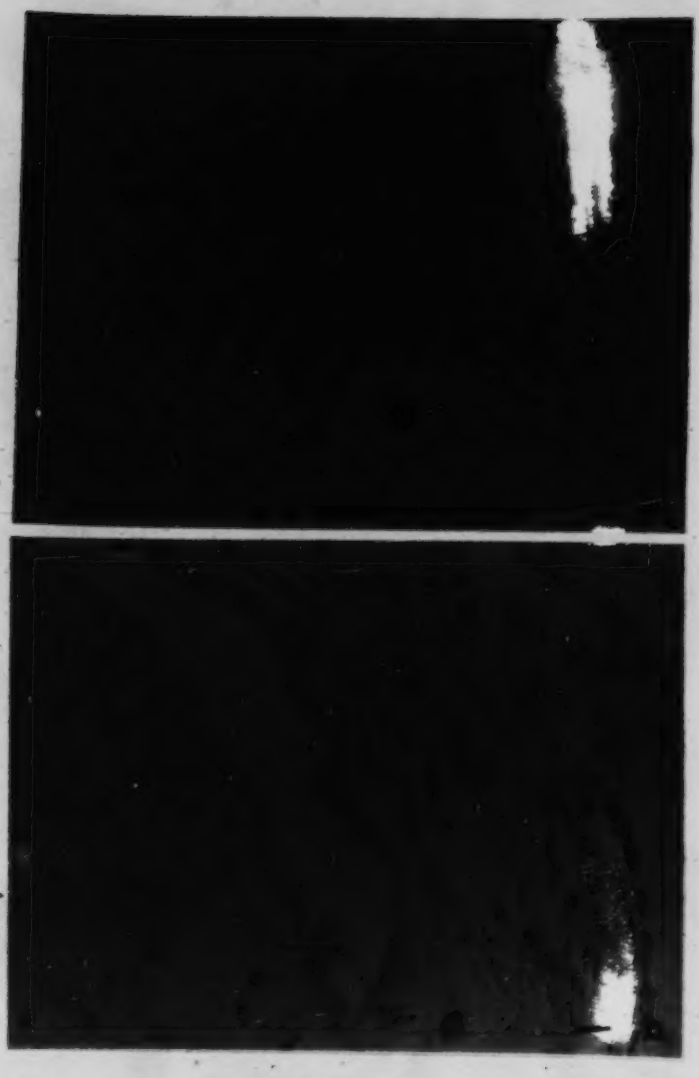


Figure 10: Specificity of the Primary Antiserum

As previously described (Fig. 9), the specificity of the primary antiserum was assessed using a different antigen with the primary antiserum and also a different Mycoplasma antiserum with U. urealyticum (Ch. II., Sect. 2.5.4., pg.41). The positive control showed that the staining procedure did work when the primary antiserum was used with its antigen (arrow). X640. The bar represents 10 μm.

works on the basis of the ability of strain specific antiserum, in the presence of complement, to inhibit the hydrolysis of a specific substrate by an homologous strain (e.g. Robertson and Stemke, 1979). The results of the metabolic inhibition test indicated that there was a significatnt difference in titre between the absorbed and non-absorbed antisera. The titre for the primary antiserum was greater than 10,240 where as the titre for the absorbed antiserum was <80. When serotypes 1 and 8 were used with both antisera, their titres were <80 (Table 7). There was no difference in titre however, when the amount of antigen for absorbing was increased. Thus, the initial amount of antigen was used to absorb further antisera.

5.1. ImmunoAssays on Infected Monolayers

5.1.1. Immunofluorescence

Confluent monolayers were infected with U. urealyticum (Ch. II., Sect. 3.3., pg.44) and stained by immunofluorescence (Ch. II., Sect. 3.4., pg.45). The attachment of U. urealyticum to the HeLa cells showed patterns similar to those described by Alfa (1986). The infected monolayers which had been incubated with anti-U. urealyticum exhibited bright green fluorescent particles on the HeLa cells when examined at 400X with the fluorescent microscope (Fig. 13). The normal rabbit serum control (Fig. 11), the monolayer incubated with anti-M. hominis antiserum (Fig. 12) and the absorbed antiserum control (Fig. 13) were all negative for fluorescence.

Table 7: Metabolic Inhibition Test using Absorbed and Non-Absorbed Antisera

Antigen Serotype	Titre	
	U. urealyticum antiserum	Non-Absorbed antiserum
1	<80	<80
3	>10,240	<80
8	<80	<80

Figure 11: Immunofluorescence on U. urealyticum Infected Monolayers

HeLa cell monolayers were infected with U. urealyticum and stained by immunofluorescence (Ch. II., Sect. 3.3., pg. 44)). Monolayers incubated with normal rabbit serum in place of the primary antiserum (a) were negative when examined under the fluorescent microscope. Examination of the monolayers with transmitted light revealed the HeLa cells were present on the coverslips (b). 640X. The bar represents 10 μm.

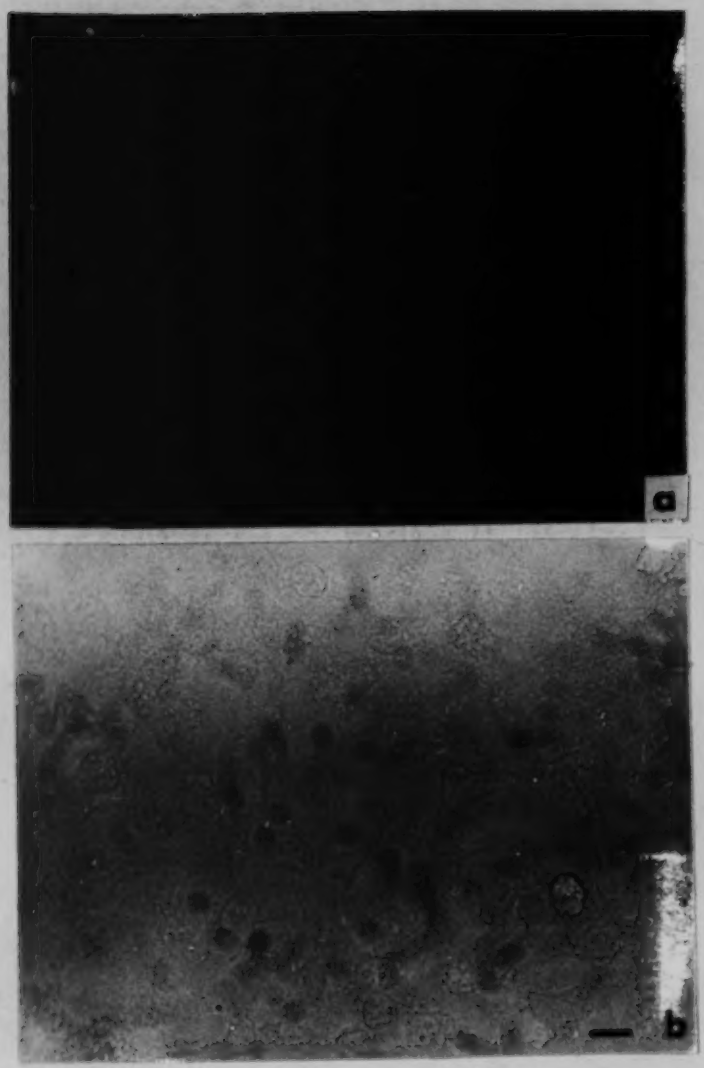

a
b

Figure 12: Immunofluorescence of Monolayers Infected with U. urealyticum

U. urealyticum infected monolayers were stained as described in the preceding micrographs (Figure 12). Monolayers were incubated with absorbed antiserum in place of the primary antiserum. Examination with the fluorescent microscope did not show any bright green fluorescent particles (a). When transmitted light was used to examine the monolayers, HeLa cells were present on the coverslips (b). X640. The bar represents 10 μm.

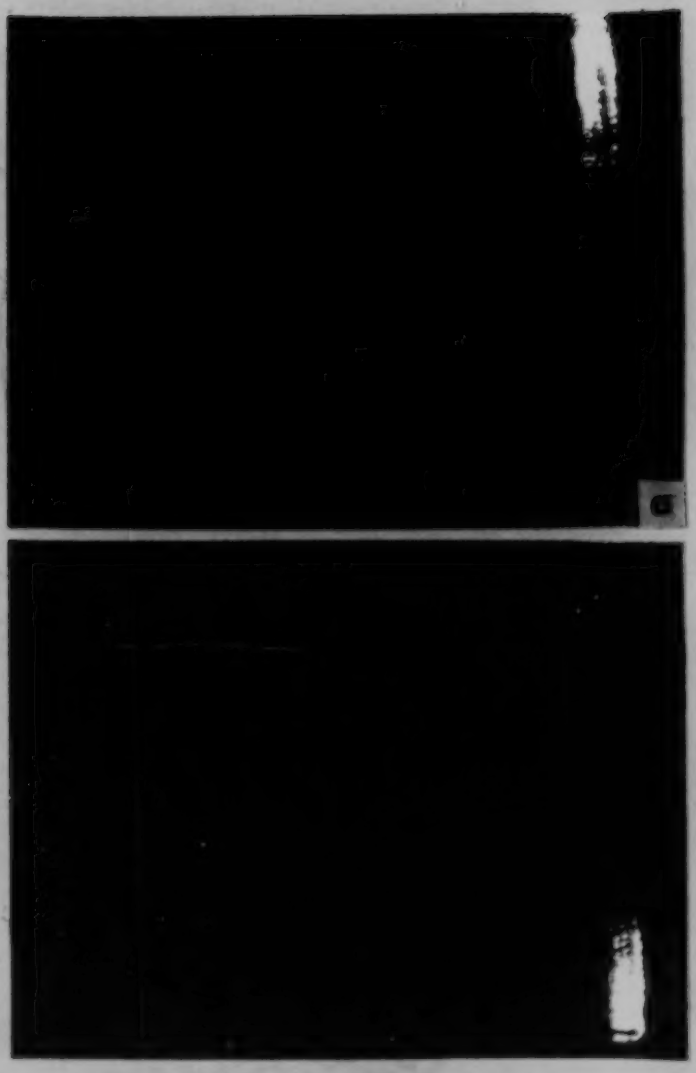
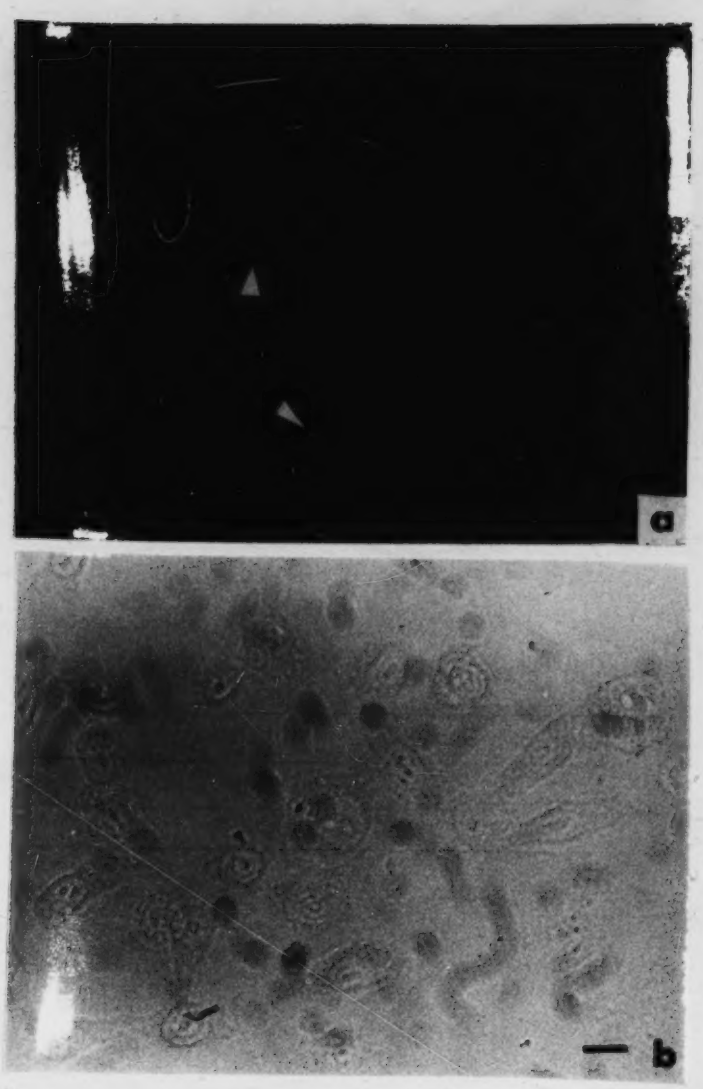


Figure 13: Immunofluorescence using U. urealyticum Infected HeLa Cell Monolayers

U. urealyticum infected monolayers were stained by immunofluorescence as described by Alfa (1986), (Ch. II., Sect. 3.4., pg.45). Bright fluorescent particles (arrows) were obvious when the infected monolayers were stained using anti-U. urealyticum antiserum (a). When transmitted light was used to examine the monolayers (b), HeLa cells were apparent where the particles were located. X640. The bar represents 10 μm.


a
b

5.1.2. Immunoperoxidase

U. urealyticum cells were attached to HeLa cell monolayers (Ch. II., Sect. 3.3., pg.44) and were stained by immunoperoxidase (Ch. II., Sect. 3.5., pg.45). The staining acheived with immunoperoxidase was similar to that of the immunofluorescence. Brown, ovoid particles associated with the HeLa cells were apparent in those monolayers stained using the primary antiserum (Fig. 15). Monolayers stained with normal rabbit serum, M. hominis antiserum and the absorbed antiserum all were negative (Fig 14). As expected, the non-infected monolayer control was also negative (Figs. 15 and 16).

6.1. Immunoperoxidase Staining on Fetal Tissues for U. urealyticum Antigen

7.1.1. On Cases Known to Contain U. urealyticum

Cases of pregnancy wastage were selected based on the presence of inflammation and the presence of U. urealyticum upon culturing (Ch. II., Sect. 2.5.1., pg.39). Staining was carried out as described (Ch. II., Sect. 2.6.1., pg.42). Examination of the tissues under the LM revealed that ureaplasma antigen was present in most tissues. The antigen was localized at various levels in the fetus, such as lung, placenta, free membranes and umbilical cord. Figs 16, 17, and 18 illustrate the presence of organisms in the fetal tissues. Table 8 shows the distribution of the organisms through the tissues stained compared to those results obtained by cultivation.

Figure 14: Immunoperoxidase Staining on HeLa Cells Infected with U. urealyticum

HeLa cell monolayers were infected with U. urealyticum and stained by immunoperoxidase (Ch. II., Sect. 3.5., pg.44). Controls of non-infected monolayers (a) and the absorbed antiserum in place of the primary antiserum (b) did not contain any brown precipitate. X1600. The bar represents 10 μm.

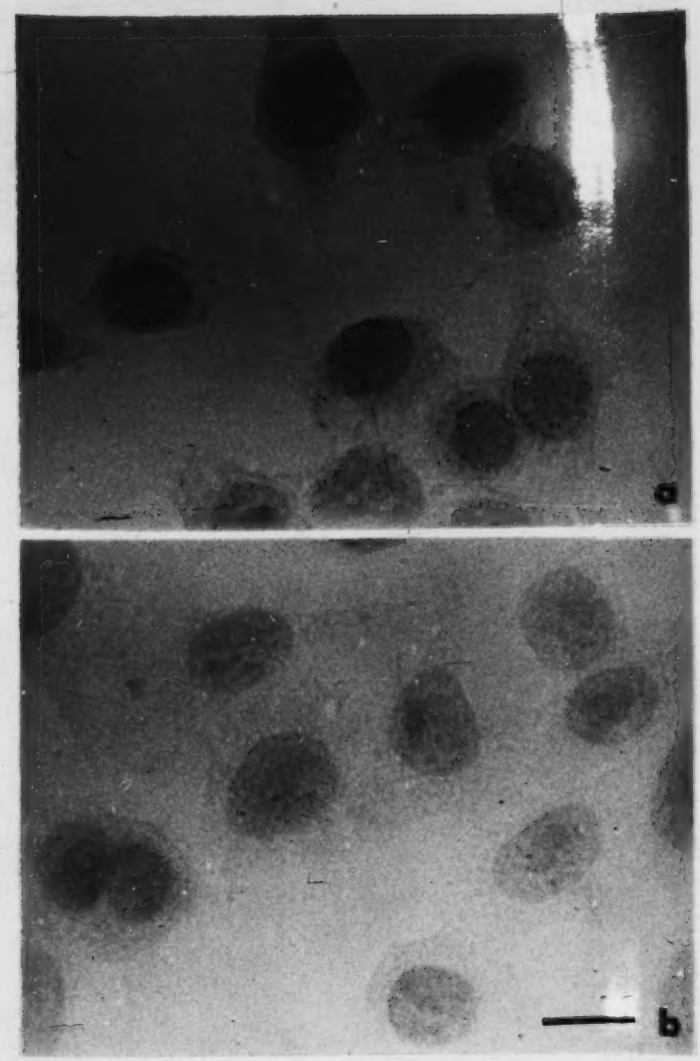

a
b

Figure 15: Immunoperoxidase Staining on U. urealyticum Infected Monolayers

HeLa cell monolayers were infected with U. urealyticum and stained with immunoperoxidase (Ch. II., Sect. 3.5., pg.44). Monolayers incubated with the antiserum raised against M. hominis were devoid of any dark brown precipitate (a). However, infected monolayers which had been exposed to anti-U. urealyticum antiserum did reveal dark brown particles (arrows) on the HeLa cells (b). X1600. The bar represents 10 μm.

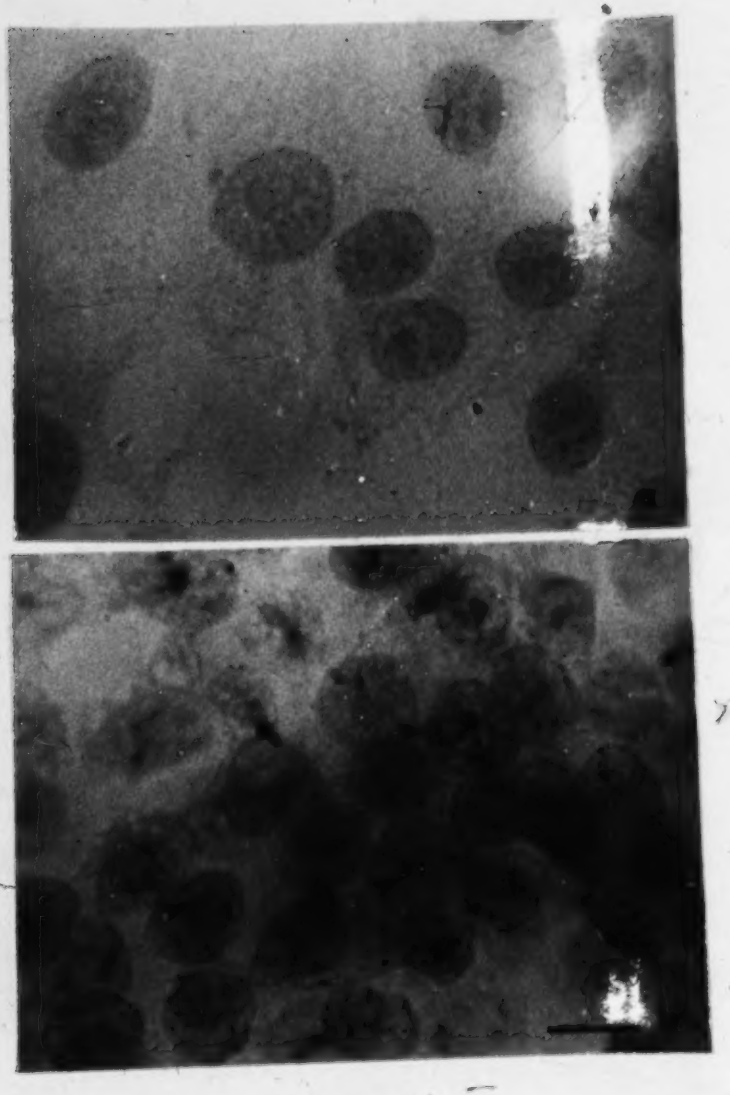


Table 8: Examination of Immunocytochemical and Microbiological Results in Cases of Spontaneous Abortion Known to Contain Ureaplasma

Tissue	454		457		507		531		533	
	C	IP	C	IP	C	IP	C	IP	C	IP
Membrane	–	–	+	+	+	+	+[3]	+	+	–
Cord	+[1]	+	+[2]	+	+	+	+[4]	–	+	–
Placenta	–	+	+	–	+	–	+	+	NA	–
Lung	+[4]	+	+	+	NA	NA	+	+	+	–

Immunoperoxidase (IP), Culture (C), Not Available (NA)

1. No inflammation was present. There was coarctation in the cord.

2. No inflammation was present. A true knot in the cord was evident.

3. An infarction was present in the placenta and adjoining membranes with secondary infection.

4. No inflammation was evident.

Figure 16: Immunoperoxidase Assay on Case 457

A normally structured, congested female fetus from a spontaneous abortion at 13 weeks showed acuted subchorionic intervillositis with patchy chorionitis and focal amnionitis. Upon staining with immunoperoxidase for U. urealyticum, brown precipitate was apparent in the placenta (arrows) with a marked inflammatory response (a). The fetal lung showed the characteristic staining for ureaplasmas (b) and the lung also contained degenerating polymorphs indicative of orificial spread of infection. X640. The bar represents 10 μm.

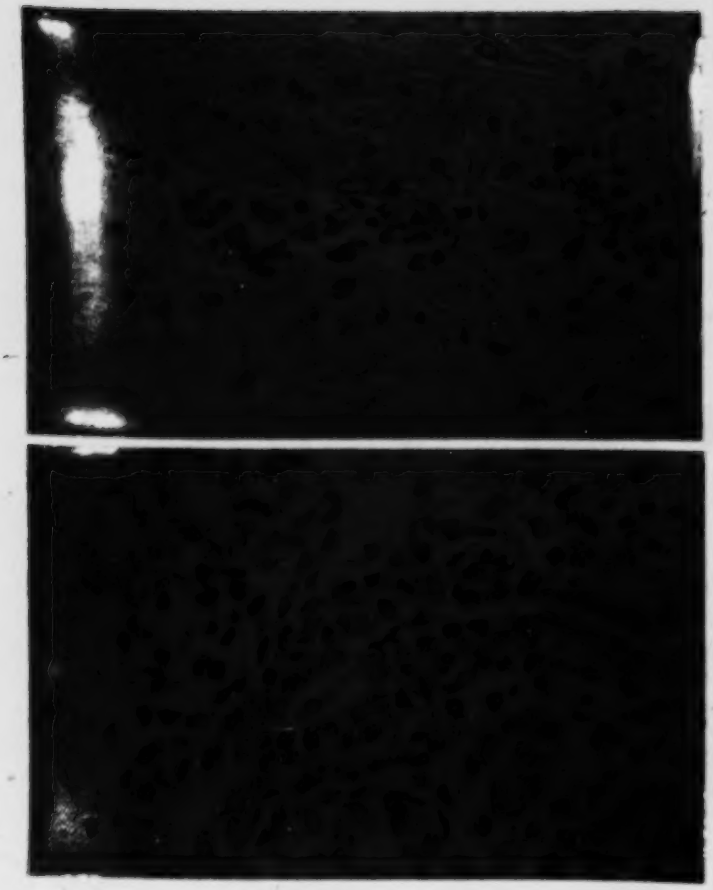


Figure 17: Immunoperoxidase Assay on Cases 454 and 507

Case 454 was a male fetus from a late spontaneous abortion. An inflammatory reaction was seen in the free membranes, chorionic plate and the maternal decidua. The fetal lung showed brown precipitate on the epithelial lining of the bronchi when stained by immunoperoxidase (a). However, no maternal polymorphs were obvious in the lung.

Case 507 was a preterm delivery of a female fetus. There was widespread inflammation in the membranes and chorionic plate. The umbilical cord showed severe vasculitis and necrotizing acute funisitis. U. urealyticum was detected in the cord by immunoperoxidase (b). Arrows indicate the characteristic brown staining particles. X640. The bar represents 10 μm.

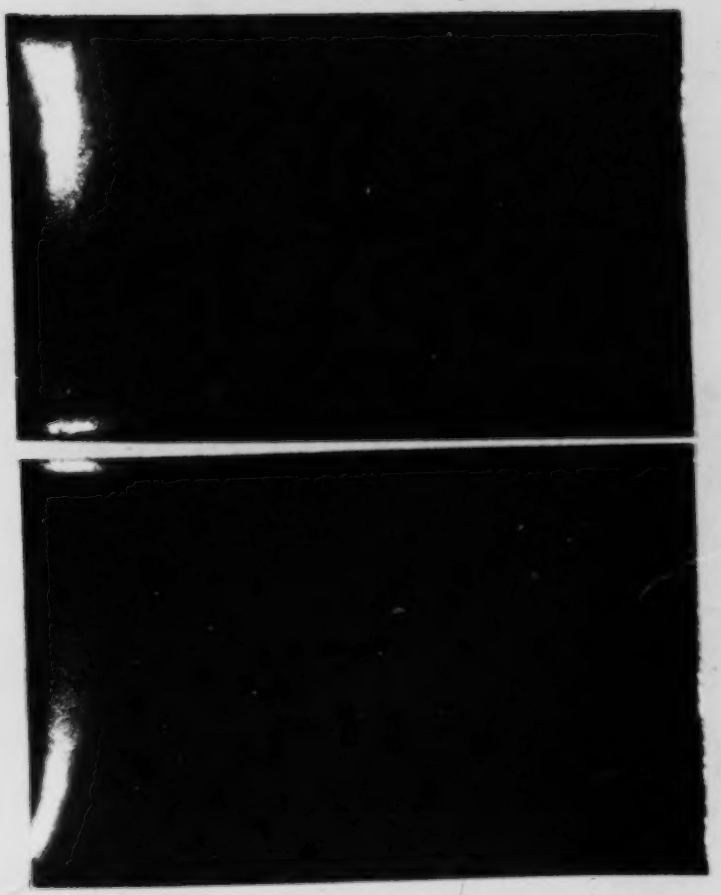


Figure 18: Case 531: Immunoperoxidase Staining of the Fetal Membranes and Umbilical Cord.

A stillborn baby boy was delivered and exhibited congenital pneumonia and chorioamnionitis. Fetal membranes were positive when stained with the immunoperoxidase method (a). The umbilical cord was also positive (b). Arrows indicate the typical pattern of the immunohistochemical staining. X640. The bar represents 10 μm.

6.1.2. Investigation of Fetal Tissues for U. urealyticum Antigen

Cases of spontaneous abortion and therapeutic abortion were selected as outlined (Ch. II., Sect. 2.6.2., pg.43). Tissue sections were stained by the immunoperoxidase assay (Ch. II., Sect. 2.5.2., pg.41). The stained sections were examined under the LM for U. urealyticum antigen. The investigator examining the slides had no prior knowledge of the results of the microbiological examination of the tissues. The staining results of the 52 blocks are presented in Table 9. Staining was compared with microbiological data using the Chi-square analysis. There was complete agreement between the microbiological data and the immunoperoxidase staining in 32/52 (62%) of the tissues. The p value was found to lie between 0.1 and 0.05, indicating a significant relationship.

The presence of inflammation in the tissues was examined under the LM. Inflammation was present in 4/51 cases which were both culture positive and immunoperoxidase positive (Table 10). A Chi-squared analysis was used to determine if the presence of inflammation was related to immunoperoxidaase staining or to microbiological results. The p values were between 0.1 and 0.25 for inflammation vs culture techniques and between 0.25 and 0.75 for inflammation vs immunoperoxidase staining. Thus, there was no significant relationship between the presence of the organism and an inflammatory reaction.

The sensitivity of the immunoperoxidase staining relative to the culture results was determined by calculating the false negative results. Twelve of the 52 blocks were negative with immunoperoxidase staining. this corresponded to a sensitivity of 77%. If the data are compared not to

Table 9: Relationship between Cultivation of Ureaplasma from Fetal Tissues and the Localization of Antigen in the Tissue

Culture	Immunoperoxidase	No.		%
+	+	16		
			32	62
-	-	16		
+	-	12		23
-	+	8		15
		52		

$0.1 > p > 0.05$

Table 10: Relationship between Culture, Immunoperoxidase Staining and the Presence of Inflammation within Tissues of Pregnancy Loss

Culture	Immunoperoxidase	Inflammation	No.	%
+	+	+	4	
			16	31
-	-	-	12	
+	-	-	8	16
+	-	+	4	8
+	+	-	12	23
-	+	+	3	16
-	+	-	5	10
-	-	+	3	16
			51	

culture but to the total number of positives for either test, then the sensitivity for cultivation is 78% (100 x 28/36) and 66% (100 x 24/36) for immunoperoxidase staining.

CHAPTER FOUR

DISCUSSION

Before applying the immunoperoxidase method to clinical tissues, certain technical aspects had to be considered. These aspects will be discussed first.

Through logical progression in the application of the PAP methodology most problems encountered were overcome. Problems included non-specific staining of smears of U. urealyticum using normal rabbit serum as a control (Ch. III., Fig. 1a, pg.49) and non-specific staining of smears of broth components using the primary antiserum (Ch. III., Fig. 2b, pg.52). Background staining was also apparent in normal rabbit serum control in fresh frozen sections (Ch. III., Fig. 3a, pg.55). Staining of the broth smears may have resulted from antibodies in the polyclonal antiserum directed against components of the broth medium used for cultivation of the organism. Alternatively, the staining might have been the result of non-specific binding of the primary antiserum to broth constituents. The problem of non-specific staining with the normal rabbit serum and the primary antiserum was overcome by increased washing times and improved technical skill of this investigator. Intense non-specific background staining can result from too high a concentration

of the primary antiserum (Bigbee et. al., 1977) and in the present study, the use of the primary antiserum at higher dilutions did decrease background staining (Ch. III., Fig. 7b & 8, pgs.68 & 70). The concentration of the primary antiserum and the amount of background staining were directly related, that is, when the primary antiserum concentration decreased, so did the amount of background staining while the specific staining increased.

The specificity of the reaction was tested in several ways. First, the primary antiserum was absorbed with its specific antigen to remove "specific" antibodies (Ch. II., Sect. 2.1.2., pg.33). The effect of the absorption procedure was determined by using the metabolic inhibition test (Ch. II., Sect. 2.1.3., pg.34) and the use of the absorbed serum in both immunoperoxidase and immunofluorescence staining of ureaplasma-infected HeLa cell monolayers. The titres of U. urealyticum between the absorbed and non-absorbed antiserum differed, as they were <80 and 10,240 respectively, indicating that most antibodies against U. urealyticum strain 27 had been removed by absorption. This effect was further substantiated by the negative results that were obtained when infected monolayers were incubated with the absorbed primary antiserum (Ch. III., Figs. 13 and 15b, pgs.82 &87). When the absorption step was proven effective, it was then used as a negative control serum when "staining" the tissues from spontaneous abortions and therapeutic abortions being investigated for U. urealyticum antigen. In no instance was this control positive.

The specificity of the primary antiserum was further tested by replacing the antigen, U. urealyticum, with M. hominis. M. hominis is another mycoplasma commonly found in the female genital tract (Holmes, 1984). Incubation of the anti-U. urealyticum antiserum with tissue injected with M. hominis proved to be negative when stained with immunofluorescence and immunoperoxidase, confirming that there was no cross-reaction between these two species. The converse of the above experiment was also performed. Tissues containing U. urealyticum were stained by immunoperoxidase using antiserum raised against M. hominis and negative results were also obtained (Ch. III., Fig 15a, pg.87). To complete the assay a future consideration may be to infect monolayers and tissues with other mycoplasmas found in the human genital tract, such as, M. genitalium, M. fermentans and ureaplasmas from other animal species, for example, U. diversum, to see if any cross-reaction of the antiserum would occur. Kotani and McGarrity (1986) have infected cell cultures with U. urealyticum, U. diversum and simian, canine and feline strains. They demonstrated the presence of the organism by DNA fluorochrome staining and by the indirect immunoperoxidase test.

A theoretical problem which I had to consider was the application of the technique using paraffin embedded tissues containing ureaplasmas. Since mycoplasmas lack a cell wall, the phospholipid membrane and extra-membranous layer are the only boundaries of the organism. Embedded within the membrane are proteins which are antigenic. Thus, with the deparaffinization of embedded tissues containing mycoplasmas, it is possible that membrane antigenicity could be altered or lost. Since Hill

(1978) was able to demonstrate M. pulmonis cells in paraffin-embedded lung using the indirect immunoperoxidase method, it was hoped that at least some antigenicity would be preserved in the tissues used in this study. Fortunately, antigenicity was preserved in the paraffin sections and false negative results did not occur in tissues infected with U. urealyticum (Ch. III., Fig. 17, 18 & 19., pgs.90, 92 &94). U. urealyticum has (extracellular) membranous and intracellular antigens, urease being one of the latter. Strain specific antigens are mostly believed to be on the membrane. It is likely that strain specificity would be lost in tissue processing and that intracellular antigens, common to all strains, would be detected. This was indirectly supported in the present study by one case of therapeutic abortion in which the organism isolated carried both the 1 and 11 determinants and immunoperoxidase positive when antiserum against serotype 3 was used. One could systematically investigate whether serotype specificity of the organism using the 14 different serotypes is lost when tissue infected with ureaplasma undergo the harsh treatment of fixation, paraffin infiltration and deparaffinization.

Localizing U. urealyticum in tissues at the light microscopic level offers an advantage over cultivation since it allows one to examine the tissue for pathological lesion and antigen simultaneously. Identifying the organism at the ultrastructural level in the tissues would be the next obvious step after this study. One problem that would arise when attempting to visualize mycoplasmas at the ultrastructural level, is the structural similarity that exists between the membranes of these prokaryotes and mammalian cells. Thus, with the advent of the

immunodiagnostic techniques at the ultrastructural levels (Sternberger, 1979; Newman et. al., 1983; Shindo et. al., 1984), it is now possible to visualize these small organisms at the electron microscopic levels. Vinther and Freundt (1979) have demonstrated M. gallisepticum using immunoferritin labelling. Visualization of U. urealyticum with immuno-electron microscopic techniques in tissue, may allow a better understanding of how ureaplasma are cause disease in the mammalian host.

In the majority of instances, U. urealyticum is identified by cultural methods. However, it is possible that the organism can be present but not viable or, the opposite, i.e. viable but not detected. In the one instance where gene probing with the whole chromosome has been reported, the probe detected U. urealyticum in 18 of 87 urethral specimens which were negative in culture and 36 of 57 speicimens which were positive in culture (Roberts et. al., 1986).

When applying the immunoperoxidase method to clinical tissues of spontaneous and therapeutic abortions, a number of factors were considered. Even though tissues taken for histopathological and microbiological study were from the same tissue, they were from adjacent sites. It was obviously impossible to have identical tissues for cultivation and immunocytochemical tests. So, a tissue which was positive for U. urealyticum in culture, may appeared negative when stained with the immunoperoxidase assay, the opposite situation could also arise. Thus, when assessing the sensitivity of the immunoperoxidase technique, a problem arises as to which method of detecting U. urealyticum, if any,

should be used as the "gold" standard. In addition to the tissues not being identical, several other factors could account for the lack of agreement between cultural isolation and immunoperoxidase localization of the organism. Differences in tissue handling could have accounted for the lack of agreement. The time between death and tissue sampling to the time they were received for microbiology varied from less than 12 hr to greater than 48 hr. The "older" tissues may have contained organisms which had lost viability over storage. We know, however, that the organism can remain viable in culture for at least 16 days at 4°C (Shepard et. al., 1974). In actuality, sometimes U. urealyticum was cultivated from tissues samples which had been held more than 48 hr before culturing (Robertson and Honore, unpublished data). Other factors which might affect the organism's viability are the substances released whilst grinding the tissues before cultivation. Grinding of the tissues was, not, however, found to effect ureaplasma viability (Robertson and Kakulphimp, unpublished data). An additional explanation for the lack of viability could be due to the result of resolving infection in which the organisms were not viable but not all antigen had yet been removed by phagocytosis. Tissue processing, such as fixation and embedding, can also change the immunoreactivity of the antigen (Pool et. al., 1983). Therefore, it is possible that antigenicity could have been lost due to these processess.

When comparing immunoperoxidase localization to cultivation, there are four possible "outcomes": those tissues that were culture positive and immunoperoxidase negative; culture negative and

immunoperoxidase negative; cultivation positive and immunoperoxidase negative and finally, culture negative and immunoperoxidase positive. Friis (1983) examined 470 pig lungs for M. hyopneumoniae by cultural and immunofluorescence techniques. Agreement between the results of the two techniques was found in 405/470 (86.2%) cases. In the present study, total agreement with immunoperoxidase and cultivation was found in 32 of the 52 (61.5%). The differences between these two studies could arise from differences in the number of organisms in the tissue, differences in the infectious process as well as the relatively small number of cases used in the present study. If the cultivation method was used as the "gold" standard, then those immunoperoxidase negative but culture positive tissues could be classified as false negative results. In the present investigation, 12 of the 52 fetal tisssues were deemed negative but were positive by culture. This corresponds to a sensitivity of 77%. Eight of the 52 tissues were positive with immunoperoxidase staining but negative in culture, corresponding to a specificity of 85%. Friis examined the sensitivity of each test relative to the total number of positives. For cultivation, the sensitivity was 87% (147/169) and for immunofluorescence, the sensitivity was 75% (126/169). Although Friis used a larger population sample, the present study had similar results. The sensitivity of the cultivation methods for U. urealyticum, relative to the total number of positives by either technique, was 78% (28/36) and 66% (24/36) for the immunoperoxidase staining.

Embree et. al. (1980) found a significant relationship between the isolation of U. urealyticum by culture, from the placenta and the presence

of chorioamnionitis. Thirty-four percent (58/170) of the inflammed membranes were culture positive for U. urealyticum. In contrast, 42 of 276 (15%) placentas with no inflammation were positive for U. urealyticum suggesting contamination of the fetal tissues with U. urealyticum. Svensson et. al. (1986) did not support Embree's findings. They investigated the role of microorganisms in the female genital tract and the presence of chorioamnionitis. These investigator cultured 86 placentas for microorganisms which have been implicated in maternal and/or neonatal disease. They found no association between the isolation of U. urealyticum from the tissues and the presence of inflammation. Although in the present study, a significant relationship was found between cultivation of this extra-cellular parasite and the localization of the antigen with immunoperoxidase ($0.1 > p > 0.5$), no significant relationship was found between cultivation of the organism or localization with the PAP technique and the presence of inflammation in the umbilical cord, free membranes, decidua or placenta. In the three tissues which showed inflammation but were negative for U. urealyticum by both method, other organisms were isolated. These included Staphlococcus, Peptostreptococcus, Bifidobacterium, Lactobacillus, Propronibacterium, Actinomyces and Streptococcus species. Thus, it is possible that these organisms could have been responsible for the inflammation found in the fetal tissues.

Animal inoculation studies have been used by several investigators to assess the role of ureaplasma in disease. Pickering (1986) found U. urealyticum to be responsible for pyelonephritis in rats and Howard et. al.

(1976) found U. diversum to cause a cuffing pneumonia in calves. Other workers have looked for animal models to investigate the role of mycoplasmas in genital infections and fetal loss. Taylor-Robinson et. al. (1975) assessed fetal wastage by intra-venous injection of mice before and after mating. They found 24 of 194 fetuses died when the mice were infected prior to mating and that 54/194 fetuses died when the mice were infected after mating. In 1978, Naot et. al. used M. arthritidis to infect pregnant rats vaginally. Both the percentage of live offspring at birth and the mean litter size were found to be lower than in the control population.

If an animal model could be developed to assess the role of ureaplasmas in pregnancy wastage, a better understanding of the pathogenicity of this organism might be attained. When developing an animal model to investigate the possible role of U. urealyticum in pregnancy wastage, several factors should be considered. If the model is hoped to parallel the human situation, it would be advantageous to use an animal with a similar reproductive physiology, such as a primate. This would, of course, be costly. It would be necessary to ensure that the animals were free of any other possible pathogens in the urogenital tract. This would ensure that if intra-amniotic infection did result, it would not be due to the other flora present in the genital tract. Since there are three methods in which intra-amniotic infection may result, inoculation of U. urealyticum in the animal model could parallel these routes. First, intra-vaginal inoculation could be used to establish an ascending infection. Secondly, intra-venous injection of the organism into the

pregnant animal would simulate hematogenous spread of the organism and finally, intra-amniotic inoculation could be used to see the effects on orificial spread and also the effects of the organism on the membranes and placenta. The adaptation of the peroxidase-anti-peroxidase method to detect the small cells of U. urealyticum in both frozen and parrafin sections is a step in the direction of elucidating the role of Ureaplasma urealyticum in causing spontaneous abortion.

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APPENDIX

Harris' Haematoxylin (Harris, 1900)

Haematoxylin (Polysciences Inc.,Warrington, Pa.)	2.5 g
Absolute alcohol (Fisher Scientific)	25.0 mls
Potassium alum (BDH Chemical Co.)	50.0 g
Distilled water	500.0 ml
Mercuric Oxide (Baker Chem. Co.,Phillipburg, N.J.)	1.25 g
Glacial Acetic Acid (AmaChem, Portland, Or)	20.0 ml

Dissolve the haematoxylin in absolute alcohol and add to the alum (which has previously been dissolved in warm distilled water). Bring the mixture rapidly to a boil and add the mercuric oxide. Rapidly cool in cold water, then add the glacial acetic acid. Filter before use.

Tris-HCl Buffer (Michaelis, 1930)

0.5 M Tris-HCl pH 7.6 (Stock)

Dissolve 242.0 g of Tris (Fisher Scientific) in 1000 mls of distilled water. Add 786 mls of 2 M HCl and bring the total volume to 4L.

0.05 M Tris-HCl (8 L)

Distilled Water	7200 mls
Tris-HCl (0.5 M)	800 mls
NaCl (Fisher Scientific) (0.9 % w/v)	72 g
Fetal Calf Serum (Flow Laboratories)	80 mls
Tween 20 (Serva) (0.05 v/v)	4 mls
2 % Sodium Azide (Fisher Scientific) (0.5% w/v)	40 mls

The pH of the buffer was adjusted to 7.6 and the buffer was stored at 4°C.

Phosphate Buffer Saline pH 7.2

Solution A (0.2 M Monobasic Sodium phosphate)

5.56 g of monobasic sodium phosphate (Fisher Scientific) was dissolved in distilled water with a final volume 200 ml..

Solution B (0.2 M Dibasic Sodium phosphate)

5.67 g of dibasic sodium phosphate (Fisher Scientific) was dissolved in distilled water to a final volume of 200 ml.

26 mls of solution A was added to 174 mls of solution B and 200 mls of distilled water was added to a total volume of 400 mls. 1.70 g of NaCl was added. The buffer was autoclaved for 20 mins and the pH was adjusted to 7.2 with sterile 1 N HCl after cooling.

H Buffer

This non-nutritive buffer was used for washing monolayers during the immunofluorescence techniques.

$MgCl_2$ (Fisher Scientific)	0.2 g
NaCl (Fisher Scientific)	5.8 g
N-2-hydroxyethyl piperazine-N'-2-ethane sulfornic acid (HEPES buffer) (Research Organic Inc., Cleveland, OH)	11.9 g
Distilled Water	1.0 l

The pH was adjusted to 6.0 using 2.0N HCl. The buffer was then sterilized at 120°C for 15 min under 15 lb pressure.

MEM Tissue Culture Medium

MEM without glutamine or sodium bicarbonate (Flow Laboratories)	2 envelopes
Ultrapure water; Milli Q Water System (Millipore)	1744 ml
Glutamine (0.2M) (Sigma Chemical Co.)	20 ml
$NaHCO_3$ (7.5 % w/v) (Sigma Chemical Co.)	30 ml
GHL Solution (20 ug/ml) (Calbiochem. Chem. Co.) (Glycl-L-Histidyl-L-Lysine Acetate)	2 ml
Penicllin/Streptomycin Solution (Sigma Chemical Co.) (100,000U/100,000ug/ml)	2 ml

The solution was filter sterilized through a 0.22 um Sterivex filter (Millipore, Bedford, Mass.). Two hundred ml of fetal calf serum (heat inactivated at 56°C for 30 min) (Gibco). The pH of the media was adjusted to 7.2 with sterile NaOH (1N).